ENZYME CATALYSIS AND PROTEIN SEPARATIONS IN REVERSE MICELLES IN NON-POLAR SOLVENTS

A Thesis Submitted
in Partial Fulfilment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

by

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to the
DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY, KANPUR
NOVEMBER, 1989

To My Guide
And To My Parents

I hereby declare that the matter embodied in this thesis entitled "ENZYME CATALYSIS AND PROTEIN SEPARATIONS IN REVERSE MICELLES IN NON-POLAR SOLVENTS" is the result of investigations carried out by me in the Department of Chemistry, Indian Institute of Technology, Kanpur, India, under the supervision of Professor S.S. Katiyar.

In keeping with scientific tradition, due acknowledgment has been made wherever the work described is based on the finding of other investigators.

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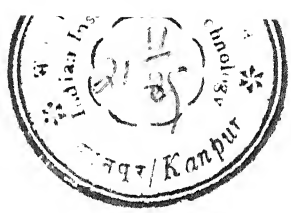

CERTIFICATE I

Certified that the work presented in this thesis entitled "ENZYME CATALYSIS AND PROTEIN SEPARATIONS IN REVERSE MICELLES IN NON-POLAR SOLVENTS" by Mr. Ajay Kumar has been carried out under my supervision and the same has not been submitted elsewhere for a degree.

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Chm	524N	Modern Methods in Physical Chemistry
Chm	525N	Principles of Physical Chemistry
Chm	542N	Advance Inorganic Chemistry II
Chm	545N	Principles of Inorganic Chemistry
Chm	632N	Enzyme Reaction Mechanism and Enzyme Kinetics
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ACKNOWLEDGEMENTS

I will remain deeply indebted to my thesis supervisor Prof. S.S. Katiyar for introducing me to a fascinating and challenging frontier area of biochemistry and for training me in many biochemical laboratory techniques. This dissertation is an outcome of his exemplary interest, continuous inspiration and dynamic guidance. I record my deep sense of gratitude for his cogent advice, personal care, encouragement and enthusiastic support throughout the work.

I am grateful to all the faculty of the Department, especially to Prof. P.C. Nigam, Prof. U.C. Agarwala,, Prof. S.K. Dogra, and Prof. P. Raghunathan for their help and encouragement.

I heartily appreciate with thanks to my lab colleagues Ms. Amita Srivastava, Dr. Anil Kumar, Dr. Manoj Srivastava, and Dr. T.K. De for their great cooperation in many ways in completion of the work for this thesis. My sincere thanks and deep appreciations are due to Ms. Manisha Borwanker, Ms. Anshu Agrawal Mr. S. Shekh, Mr. M. Shanavas, Dr.(Ms.) Valli V. Akella and Mr. Neilay Dedhia for their warm friendliness and cooperation in the moments I shared with them.

I express my thanks to Mr. Surendra Prasad, Mr. Rajendra Prasad, Dr. D.S. Pandey, Dr. G.P. Singh, Mr. Ranjeet Sarpal, Mr J.K. De, Mr. K.M. Singh, Miss Shalini Nigam, Dr. (Miss) Pratima Mishra, Miss Kavita Shah, Miss Preeti Dhar and all my colleagues for their selfless cooperation in different ways.

I am thankful to Mr. Anil Jauhari for typing the dissertation; Mr. R.K. Bajpai, Mr. J.N. Tripathi and Mr. A.K. Bhargava for tracing the figures, Mr. S. Pandey, Mr. J.S. Rawat for procuring the chemicals and equipments. The help by M/s T.G. Rao, R.K. Jha, U.N. Pandey and many others of the Institute are sincerely acknowledged.

I express my thanks to Mr. H.N. Singh for his cordial relation and assistance.

I am highly grateful to Prof. M. Prasad and his family members for their generous help throughout my stay.

Finally, I record my profound sense of gratitude to my parents and other family members for their exemplary patience, understanding and cooperation. Their sacrifice in various ways for my endeavour is gratefully acknowledged.

AJAY KUMAR

SYNOPSIS

Enzymes are responsible for catalyzing almost all the chemical reactions in biological systems. Besides their role in living system, enzymes have great potential for application in a wide range of fields which is largely unrealized. The specificity and high catalytic activity of enzymes is being exploited for the syntheses of important bioactive materials and medicinal compounds. However, wider potential application of enzymes and other proteins in biotechnology is restricted due to the following major problems: (i) the separation and purification of proteins at industrial levels. (ii) the stability of these proteins/enzymes in non-polar organic solvents.

First focus of the present work was to study the separation of proteins/enzymes by a novel technique of separation using reverse micelles in apolar solvents. Traditional separation methods like chromatography, electrophoresis and affinity chromatography are mostly limited to laboratory-scale analytical applications. Extraction of proteins/enzymes and other biomolecules by organic solvents containing reverse micelles is a relatively new concept. The method fulfills the need of an extracting phase which does not harm enzymes and other labile proteins. This promising technique is based upon the selective and controlled solubilization of proteins and other bio-compounds in reverse micellar organic phase, and has to be developed into a liquid-liquid extraction technology for bioseparation.

The next focus of the work was on the catalytic efficiency

of enzymes in non-polar organic solvents. While enzymes in general are employed in aqueous media, several interesting applications of enzyme catalysis in organic solvents are not feasible due to their denaturation or appearance of undesired substrate specificity in organic solvents. Study of enzymes in vitro are usually conducted in aqueous solution, where the environment is different than the natural environment of the cell. The solubilization of enzymes in reverse micelles in organic solvents probably provides an environment which is somewhat similar to cellular medium.

The thesis consists of four chapters, each centers around a different aspect of the above mentioned work. Chapter I presents the introduction and background with pertinent literature of the work in the area of micelles, reverse micelles and their biotechnological applications. Exploitation of reverse micelles for the solubilization, stability of proteins and kinetic investigations of enzymes in reverse micellar non-polar media has been reviewed in detail. The new concept of liquid-liquid extraction for the recovery, separation and purification of proteins/enzymes using reverse micelles has been briefly discussed.

Chapter II reports the data on the solubilization of proteins by the following techniques; (i) liquid-liquid phase transfer of proteins, and (ii) solid extraction of protein from the dry powder. The influence of structural parameters of the proteins and reverse micelles as well as the effect of external

parameters such as pH, salt concentration on the specificity of solubilization processes of proteins have been investigated.

The solid extraction of proteins (pepsin M_r 35 kDa, BSA M_r 65 kDa and lipase M_r 100 kDa) was examined in cationic reverse micelles of 0.1M CTAB/$CHCl_3$-isooctane (1:1, v/v) and anionic reverse micelles of 0.1M AOT/isooctane, as a function of W_o([H_2O]/[Surfactant]), pH and concentration of surfactant. The maximum solubilization of pepsin, BSA and lipase in AOT/isooctane occurred at W_o values of 6, 11 and 8 respectively. In cationic reverse micelles of CTAB/$CHCl_3$-isooctane, it was at W_o 20, 16 and 8 respectively. Protein solubilization profile as a function of pH showed a maximum at the pH near the isoelectric point (pI) of proteins. At fixed W_o and pH of the waterpool, solubilization increased with increasing concentration of the surfactant.

The forward and backward transfer of proteins/enzymes and biomolecules using "water-reverse micellar organic phase" liquid system have been investigated in 50 mM AOT/isooctane system. The effects of pH and salt concentration on the solubilization of cytochrome-c (M_r 12 kDa), pepsin (M_r 35 kDa), creatine kinase (M_r80 kDa), lipase (M_r 100 kDa, cytochrome-c reductase, (M_r 80 kDa), yeast alcohol dehydrogenase (M_r 140 kDa) and other coenzymes have been studied to explore the potential for employing the phase transfer techniques in the large scale separation and concentration of proteins using liquid phase extraction. Creatine kinase, a relatively bigger protein shows complete solubilization in 50 mM AOT/isooctane, whereas BSA (M_r

65 kDa) does not solubilize at any pH. The complete insolubilization of low molecular weight coenzyme NADH and lipoic acid at some conditions shows that solubilization of proteins and other biomolecules is governed by electrostatic interaction as well as the size effect. Above the pI, the protein solubilization is diminished because of unfavourable electrostatic repulsions between the like charged proteins and surfactant heads.

The solubilization characteristics of single proteins have been used to separate a series of binary mixtures like cytochrome-C + lipase, cytochrome-C + creatine kinase, creatine kinase + lipase and creatine kinase + NADH etc. and ternary mixtures like creatine kinase + BSA + lipoic acid and cytochrome-C + creatine kinase + lipase etc. A relatively more difficult separation was achieved from the quarternary mixture of cytochrome-C + creatine kinase + lipoic acid + BSA. In this case, both the size exclusion factor and differences in the electrostatic interactions were exploited.

Chapter III reports a detailed and systematic investigation on the activity, stability and kinetic characteristics of flavo-enzyme glutathione reductase from yeast and bovine intestinal mucosa sources *in vitro* in CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) reverse micellar solution. It is a dimer with one FAD per monomer of 50 kDa. The scheme of the catalysis is shown below:

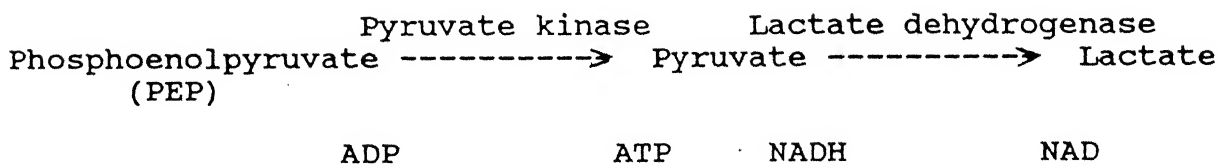
$$GSSG + 2\ NADPH \xrightarrow[\text{reductase}]{\text{Glutathione}} 2\ GSH + 2\ NADP^+$$

The study of the enzymic reduction of disulfide bond presents a new class of investigation in reverse micellar medium. The activity of the enzyme was notably affected by the change in W_o, pH and concentration of surfactant. Maximum activity was shown by the yeast enzyme towards the lower pH and higher W_o values. The highest activity of glutathione reductase was achieved at a value of W_o = 29.7 and pH 8.0. At pH > 8.0, the enzyme exhibited more activity in reverse micelles than that in water at the corresponding pH value.

The spectral features of enzyme system containing reverse micelles were similar to those observed in aqueous medium. Emission spectra of glutathione reductase in both media revealed that the position of tryptophan residues in the 3-dimensional conformation of enzyme is intact. These observations show that glutathione reductase maintains its native conformation in micro-heterogeneous medium. The enzyme followed Michaelis-Menten kinetics in reverse micellar media. Substrate inhibition was observed when concentration of one of the substrates, GSSG or NADPH was present in large excess over the other substrate. The nature of double reciprocal plots suggested that glutathione reductase from yeast follows the sequential mechanism in both the aqueous and reverse micellar media. The K_M values for GSSG and NADPH in CTAB/H_2O/$CHCl_3$-isooctane system determined at various W_o values are in the range 40-60 μM for GSSG and 5-25 μM for NADPH, which are quite close to the K_M values obtained in the aqueous medium.

The storage stability in CTAB/H_2O/$CHCl_3$-isooctane reverse micelles at W_o 29.7 and pH 8.0 indicated that glutathione reductase (yeast) retained ~80% of its activity for a period of one month. Oxidized glutathione (GSSG) provides protection to the enzyme against denaturation, probably by shielding the exposed site of glutathione reductase against organic solvent. Apparently, glutathione reductase entrapped inside the cationic reverse micelles in $CHCl_3$-isooctane (1:1, v/v), under some conditions, exhibits activity and stability almost comparable to that in aqueous solution.

Chapter IV presents investigations on the activity of a combined enzyme system, pyruvate kinase and lactate dehydrogenase in the solvent system $CHCl_3$-isooctane (1:1, v/v) in presence of cationic surfactants cetrimide (mixed alkyltrimethyl ammonium bromide). The enzyme from rabbit muscle is tetrameric (subunit M_r 57 kDa) having overall molecular weight 240 kDa with four metal binding sites. On the other hand lactate dehydrogenase is comprised of four subunits with a total molecular weight 145 kDa. Despite the large molecular weight and increased complexity, these enzymes are able to catalyze the usual concerted reaction in non-polar reverse micellar media.



In the reverse micellar solution of cetrimide in $CHCl_3$-isooctane, an induction period similar to that in aqueous medium

was observed. The length of this induction period was determined by the parameters of the second step reaction catalyzed by lactate dehydrogenase (reduction of pyruvate to lactate). The pyruvate kinase in this reverse micellar system exhibits super activity (i.e. the activity in organic medium is greater than that of aqueous solution under optimum conditions). Waterpool size, pH and surfactant concentration remarkably affected the activity of the combined enzyme system. The enzyme system exhibited maximum activity at water content ~3 to 4%, pH 7.8 at 4% cetrimide in $CHCl_3$-isooctane (1:1, v/v). The identical nature of the enzyme reaction in both the organic and aqueous media was established with the help of electronic spectra. The effect of concentration of the substrates phosphoenolpyruvate, ADP and enzyme, pyruvate kinase in reverse micelles were investigated on the rate of overall reaction. These observations demonstrate that the enzyme system obeys Michaelis-Menten kinetics in the organic media. The kinetic parameters such as K_M and turnover numbers at different conditions were determined.

In summary, the investigation of the catalytic role of glutathione reductase and that of combined enzyme system (pyruvate kinase + lactate dehydrogenase) in organic micellar media, is a major step towards the understanding of experimental techniques for carrying out multi-step enzymic catalysed reactions in organic solvents. These studies provide optimism for the use of reverse micelles as a versatile medium for a carrying out chemical transformations catalyzed by enzymes in organic solvents.

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CHAPTER I

GENERAL INTRODUCTION

Living cells function as "self regulating chemical engines" because they contain enzymes. Enzymes are the biological catalysts possessing enormous catalytic power and substrate specificity. Unlike the synthetic catalysts, enzymes in general are more fragile. It is relatively easy to disturb the native conformation and destroy the enzyme activity. Enzymes can function only under mild conditions i.e. moderate temperature, physiological pH etc. in comparison to rather extreme conditions often required for nonenzymic reactions. A thorough and in-depth understanding of their function and regulation are of immense importance towards an understanding of biological processes.

I.1 Potential of Enzymes in Biotechnology

Enzymes have tremendous potential for application in a wide range of fields. Industrial exploitation of the high substrate specificity and extraordinary catalytic power of enzymes under mild conditions has assumed great importance for the synthesis of commercially important bioactive materials, medicinal compounds and wide variety of substances used for research in biochemistry,

metabolism and pharmacology. The major applications of enzymology in industry lie in the successful use of enzymes in the manipulation of sugars, nucleic acids, amino acids and lipids [1]. Enzymes play an increasingly important role in modification of materials derived from fermentation synthesis [2]. Peptidases are used to cleave or to form selective peptide links and other enzymes to attach oligosaccharides moieties to proteins or to modify those of glycoproteins. In particular, amylases are extensively applied enzymes which can hydrolyze the glycosidic bonds in starch and related glucose-containing compounds, by reducing the solution's viscosity. They are used in distilleries and in the manufacture of glucose syrups (corn syrup) and crystalline glucose. Uses of free proteases occur in dry-cleaning detergents, meat processing, cheese making (rennin only), tanning, silver recovery from photographic film (pepsin), production of digestive acids, and in certain medical treatments of inflammations and virulent wounds. The use of proteinases in the food industry has a long history. For example, pectic esterase enzyme is used for the manufacture of jelly from juices. The recent commercialization of a process for conversion of porcine to human insulin by Novo Industries, U.S.A. provides the first large-scale utility. Several large-scale industrial processes already in operation employ immobilized-enzyme catalysts at some point. Two notable examples are production of high fructose syrups from corn starch and manufacture of L-amino acids by resolution of racemic amino acid mixtures (containing both D & L isomers). Given below are several areas in which a potentially

exciting future may be identified.

(1) Bioprocess Design

Optimization of biocatalysts by genetic engineering, selection of thermostable systems or use of immobilized configurations; development of cheap, stable, efficient and generally applicable methods of cofactor recycling; chemical engineering in relation to large scale biocatalytic system.

(2) Reaction Configuration

Use of common enzymes for unexpected chemistry; protein engineering to alter catalyst properties; use of biological catalysts or protein design for non-aqueous media.

For our study, two technologically important fields have been chosen; (i) the separation and purification of enzymes/proteins and other biomolecules at industrial level and (ii) the study of the water soluble/hydrophilic enzymes in non-aqueous solvents.

Most of the recent studies in the biotechnology have focussed on upstream developments and been primed by advances in genetic engineering and bioreactor design. However, successful and cost effective commercial bioprocess technology has long been hampered by nonavailability of economical, continuous, large-scale separation techniques for concentrating and separating the desired protein, enzyme or other bioproducts from complex fermentation broths and cell culture media. Traditional

separation processes such as distillation can not be used due to delicate nature of bioproducts. On the other hand methods like chromatography, electrophoresis and affinity chromatography are either limited to laboratory scale analytical application or require more expensive material like antibodies to make immunosorbant. Besides this, the isolation of specific extracellular enzymes from a fermentation broth by conventional processes consists of the stepwise removal of undesired compounds from the broth. Consequently, new isolation techniques, more selective for the required enzyme and easier to scale up, are desirable. Liquid-liquid extraction processes involving the use of reverse micelles is a relatively new concept. This can be conveniently scaled up for multi operation resolving the large product mixture and allow recovery of modesty stable whole organelles and other substructures. The promising technique is based on the selective and controlled transfer of enzymes/ proteins from one bulk aqueous phase to another in an intermediate reverse micellar phase, which has to be developed into a liquid-liquid extraction technology for bioseparation. The second chapter of the thesis presents an exhaustive study on the separation of protein mixtures by this technique.

The next problem namely the investigation of enzyme stability in artificial and suboptimal environments is a promising application in biotechnology (applied enzymology). Nature has not designed most enzymes to work in a non-aqueous environment, and as a result, many enzymes are destabilized by

the addition of organic solvents. The ability to use enzymes in a non-polar environment offers a tantalizing array of synthetic possibilities. The remarkable specificities of enzymes can be exploited in a variety of synthetic reactions but would be impractical in aqueous solution. In non-aqueous solvents, enzymes could perform biospecific transformations on the many organic compounds poorly soluble in water. Various advantages of enzymatic catalysis in organic solvents include greater enzyme stability, small reactor volumes, alteration in reaction specificity and greater ease in product recovery.

A reasonable way out of this situation is the solubilization of enzymes in organic solvents with the aid of surfactants and small amount of water (i.e. the formation of reverse micelles). In reverse micelles, the enzyme is isolated from the organic solvent by a surfactant layer, and these systems therefore show promise for combining the advantages of organic and aqueous-phase to provide a novel media for enzyme systems. This novel system has been studied in chapters III & IV in detail.

I.2 Aggregation of Surfactants

Molecules that possess both hydrophilic and hydrophobic (lipophilic) parts are termed amphiphiles or amphipathics. They are also referred to as surfactants since they absorb to surfaces and interfaces and change the interfacial free energy i.e. change the free energy associated with the building of an interface. Due to their very structure, surfactants exhibit abnormal

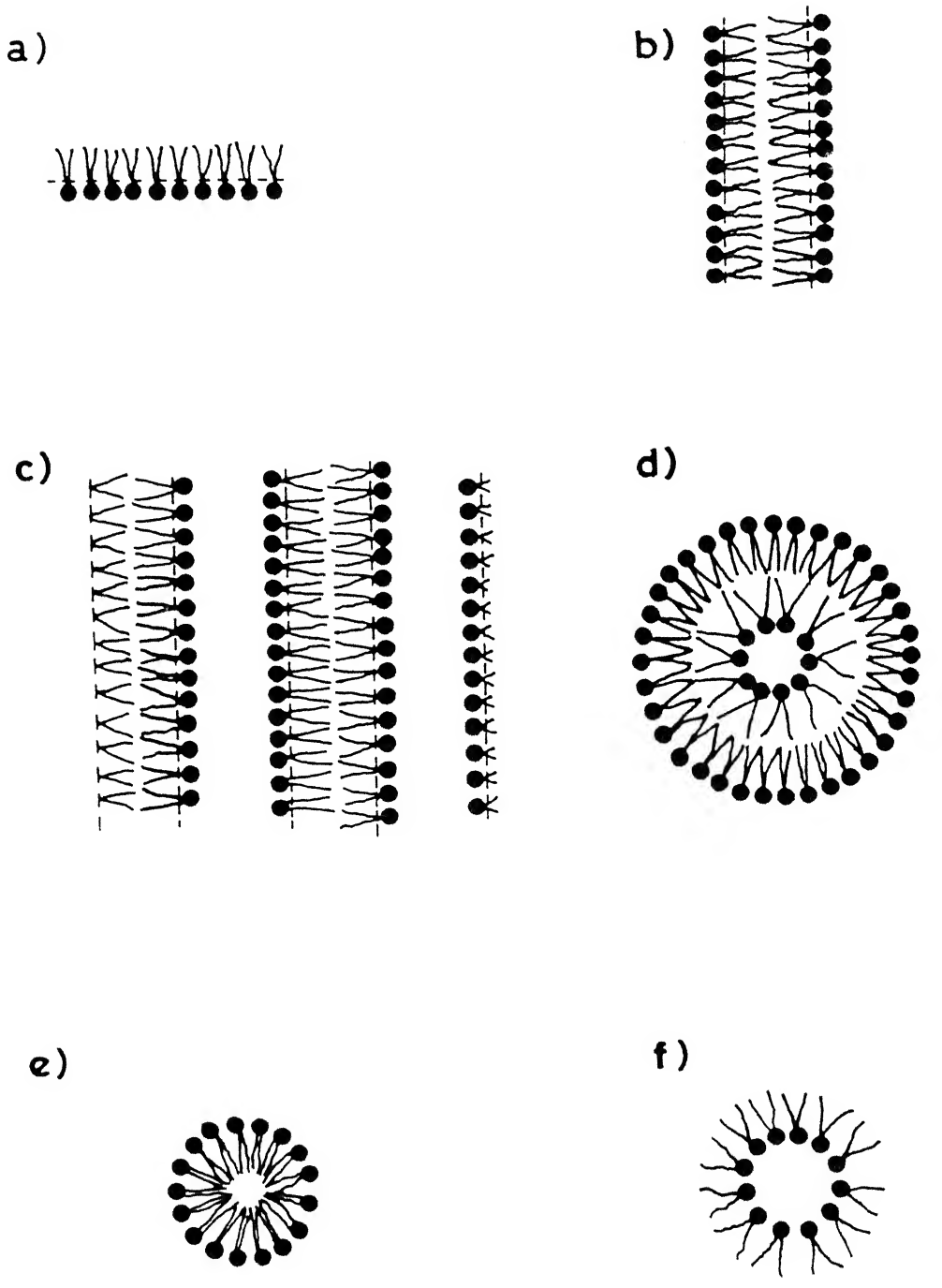

Fig. I.1. Different aggregation forms of surfactants in solution: (a) monolayer, (b) bilayer, (c) liquid crystalline phase (lamellar), (d) vesicle (liposome), (e) micelle in aqueous solution and (f) micelle in nonpolar solvent (reverse micelle).

behavior in solution i.e. aggregation of surfactants. Fig. I.1 shows the graphical representation of various kinds of aggregation of surfactants [3].

I.3 Characteristics of Aqueous Micelles

Many surfactants aggregate in aqueous solution to form the aqueous micelles. These micelles forming surfactants typically have structures which are constituted from a polar head group and a straight chain of the alkyl group usually 8-18 carbons in number [4,5]. These surfactants form micelle at concentration above the CMC (critical micelle concentration). Such micelles have average radii of 12-30 Å and contain 20-100 surfactant molecules. Micelles at concentrations close to their CMC are assumed to possess spherical and ellipsoidal structures [6]. A schematic representation of a spherical ionic micelle is shown in Fig. I.1(e). Recently an alternative model of the micellar structure has been proposed by Menger [7]. According to his "Reef Model" micelles possess rugged, dynamic surfaces and water molecules penetrate close to the micellar core. However, there are different controversies about the micellar structure [8].

I.3.1 Micellar Effect on Rate of Chemical Reaction

Aqueous micelles have shown remarkable effect on several organic reactions. It has been found that cationic surfactants increase and anionic surfactants decrease the pseudo first order rate constants for the Cannizaro reaction of benzaldehyde [9].

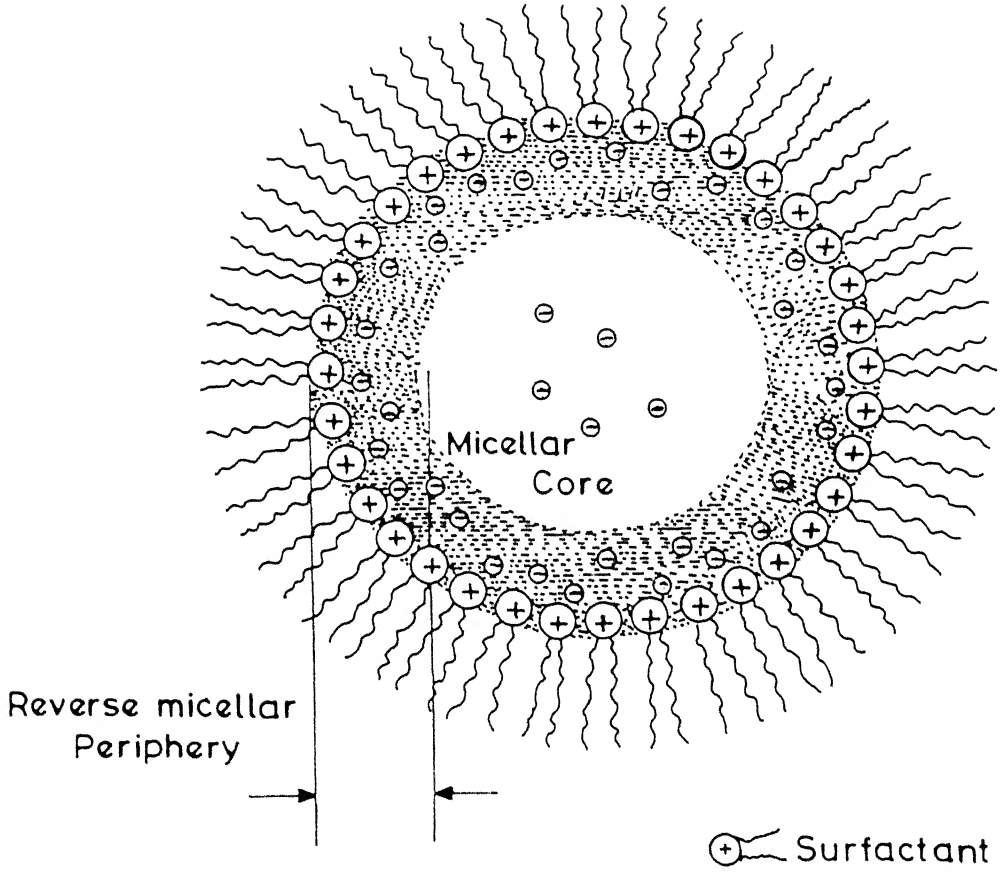

Fig. I.2. A schematic representation of spherical cross-section of an idealized cationic reverse micelle. (⊕) the polar head group, (⊖) the counterion, (∿∿∿) the hydrophobic tail of surfactant.

Other type of reactions studied in normal micelles are base catalyzed hydrolysis of α,β-unsaturated ketones, synthesis and hydrolysis of benzylidine -aniline, thiol -thiamide exchange reaction, hemin equilibria, electrophilic coupling reactions, etc. [4,10]. Transamination reactions of tetramethylazine-bis-barbiturate, barbiturylazineinandione etc. with semicarbazide have also been studied in micellar media in detail [11,12]. An interesting work has been done on the study of kinetics of triphenylmethane carbocations with several nucleophiles by Katiyar's group [13,14] and other workers [15,16]. Special attention was paid on the reactions of ethyl violet, methyl violet, malachite green, brilliant green, setoglaucin etc. with nucleophiles such as OH^- and CN^- in the presence of cationic, anionic and nonionic micelles. A theoretical treatment for micellar effects and for the effects of counterions was developed [17,18].

I.4 Reverse Micelles

Reverse micelles are formed by the aggregation of surfactants which are amphiphilic molecules in organic solvents: the polar groups (head) of the surfactant molecules are directed towards the interior of the spheroidal aggregates forming a polar core and the hydrophobic chains are directed towards the organic solvent. The polar inner core containing waterpool can host hydrophilic molecules/biopolymers/enzymes/proteins as shown in Fig. I.2. The phenomenon of aggregation of amphiphiles in

organic solvents has been recognized for several years [19], but until recently, theories and models for aggregation were based largely on those derived from aqueous micelles [4,19,20]. These aggregates are dynamic-equilibrium species. However the number of monomers involved in most of these cases is relatively small, typically less than 10 for alkylammonium carboxylates compared with up to 100 for aqueous micelles [4]. A few characteristic differences between aqueous micelles and reverse micelles are presented by Kerts et al. [21].

Aggregation of surfactant monomers in non-polar organic solvents occur because of a decrease in the overall free energy of the system. The predominant driving force for aggregation of ionic surfactants is the dipole-dipole interaction of the head groups [22]. The nature of solvent (its polarity, dielectric constant and solubility parameters) remarkably affect the extent of competition between solute-solute and solute-solvent interactions and result in a significant change in the aggregation behavior of surfactants. Interactions between the polar parts of the surfactant molecules and the solvent molecules hinder the aggregation. For ionic compounds the balance between the solubilities of the cation and anion determines the aggregation process. If both ions are large organic molecules with high solubilities in nonpolar solvent, aggregation should not occur. If one of the ions is small and relatively insoluble while the counter ion is soluble, aggregation is expected to occur. Examination of the hydrophile-lipophile balance of nonionic surfactants leads to similar conclusions.

It has been found that the association energy of reverse micelles seems to be mainly enthalpic in nature rather than entropic as in the case of aqueous micelles. The enthalpy and entropy changes of aggregation have been determined for potassium benzene sulfonate in heptane [23]. The values obtained, H= -79.5 kJ mol^{-1} and S= -62.8 JK^{-1} mol^{-1}, suggest that aggregation is primarily an enthalpy effect.

I.4.1 Characteristics of Water Inside the Reverse Micelles: Waterpool

The amount of water entrapped inside the core of reverse micelles is expressed in terms of W_o (i.e. W_o = $[H_2O]$/ [Surfactant]). The change in chemical behavior of guest molecules at low water content i.e. low W_o is characteristic of reverse micellar system. The waterpool of reverse micelles contains different water concentrations in rapid exchange with one another and is characterized by different motional properties and degree of organization. The size and shape of reverse micelles is highly dependent on the number of water molecules available per head of the surfactant molecule (W_o = $[H_2O]$/ [Surfactant]). These phenomena have been elucidated by various techniques like light scattering [24], NMR [25], ultrasound [26] or photon correlation spectroscopy [27] etc. In AOT/heptane system studied by NMR spectroscopy [28] over a large W_o range ($1 < W_o < 50$), it has been found that water is highly immobilized in the micellar interior at low W_o and the mobility increases with increasing W_o, gradually approaching that of bulk water.

Water solubilized in apolar solvents by different surfactants (cationic, anionic, nonionic/zwitterionic) exhibits the absorption bands in near I.R. spectral region (5200-4700 cm^{-1}) [29,30]. The observations have been attributed to different water populations; one bound to the surfactant polar heads and the other dispersed in the bulk phase. In spite of significant experimental data, to date, it is not possible to quantify the endomicellar water properties and to predict its behavior in different situations.

I.4.2 pH of Water in the Reverse Micellar Waterpool

The definition and the measurement of pH of the water inside the reverse micelles is difficult from the conceptual and experimental point of view. It is almost impossible to use a glass electrode in an organic solution containing as little as 1% water. pH values have been estimated by the study of acid-base indicator equilibria in reverse micelles of ionic surfactants and equations have been developed to calculate the local pH in the core of the micelles and the surfactant/solvent interface [31,32]. However, since the micellar water is a different medium as compared to bulk water, the interpretation of these measurements is questionable, because a pK_a value must be assumed which cannot be measured independently.

A new approach is to consider that an absolute determination of the endomicellar pH is impossible and therefore to base for the acidity measurements on an empirical activity scale.

TABLE I.1: pK_a Values of 3,4-DNP-OH in Reverse Micellar Solutions

AOT/Isooctane		CTAB/Isooctane-$CHCl_3$ (1:1,v/v)		E_4C_{12}/Isooctane	
W_o	pK_a	W_o	pK_a	W_o	pK_a
3	7.7	7	5.5	3	6.45
6	7.5	13.6	5.4	5	6.45
25	7.2	16.9	5.35	9	6.15
(Water)	5.15	24.7	5.35	12	6.05

Fig. I.3. Possibilities for the exchange process of guest molecules in reverse micelles. (A) exchange through diffusion process via the formation of transient dimer. (B) exchange through fusion of two reverse micelles.

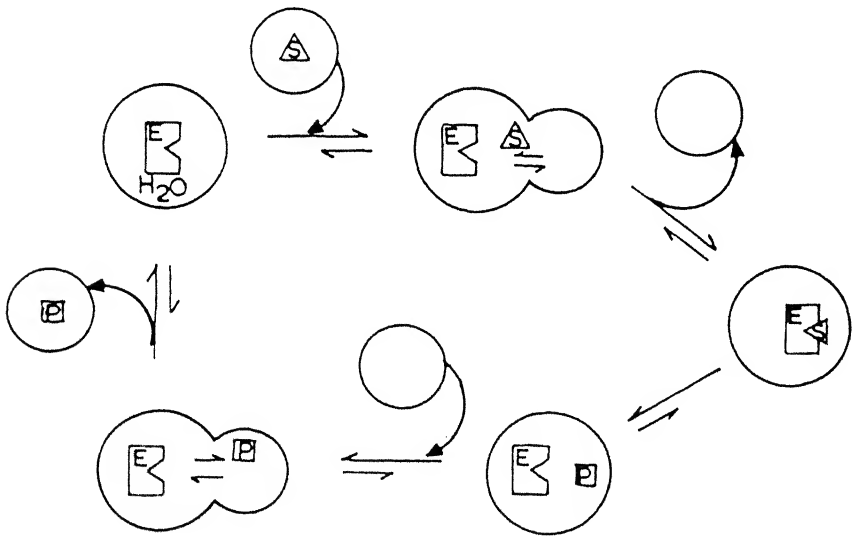

Fig. I.4. Schematic representation of an enzymatic reaction in a reverse micellar system. E, S and P represent the enzyme, the substrate, and the product, respectively. The circles represent the waterpool (cross-section) of the reverse micelle. Water is present in all reverse micelles, but is not expressly indicated for the sake of simplicity.

Assuming that the pK_a value for the dissociation of phosphoric acid is same in both waterpool of AOT reverse micelles and bulk aqueous solution, the P^{31} chemical shifts of phosphate buffer in both media have been compared to obtain such a scale [33,34]. Based on this technique, pK_a values of 3,4-DNP-OH in reverse micelles have been given in Table I.2 [34]. For the case of CTAB/$CHCl_3$-isooctane, pK_a changes are small, but in the case of AOT, changes are even larger. The interpretation of this phenomenon is not easy and clearly does not equal with simple electrostatic consideration. By this method, pH_{wp} was found to be generally within 0.5 pH unit of pH_{st} (which is pH of the solution to be solubilized).

I.4.3 Dynamics of Solubilizate Exchange Between Reverse Micelles

It has been established experimentally that while exchange of small solubilizate molecules among reverse micelles is generally much more rapid than any of the chemical reaction in which the molecules may participate (may not be true for enzymes as guests), the exchange is lower by a factor 10^3 than the diffusion-controlled rate [35,36]. In other words for 1000 micellar collisions only one collision results in solubilizate transfer. Exchange processes on time scales ranging from 100 μs to 10 ms have been reported for the Ni^{2+}/murexide ion pairs [37] and for the $TbCl_3$/phenylacetic acid pair [38] in AOT reverse micelles.

contents between waterpools. Part A shows that the two waterpools remain intact and the transfer of water and other solubilizates occur through the interfacial layers. Part B proposes the "fusion of waterpools" model, so that a single transient aggregate is produced. This model assumes that the exchange rate should be independent of solubilizate size. Fig. I.4 is the simple representation of this situation in the case of an enzymatic reaction taking place in the micellar organic phase, in the simple case in which the enzyme E and the substrate S are only soluble in the waterpool. The active E-S is formed when a enzyme containing reverse micelle collides and partly fuses with the E-P containing micelle.

I.4.4 Reverse Micelles as Novel Microreactor for Chemical Reactions

The effect of reverse micelles on the rate of various organic and inorganic reactions in non-polar solvents has been extensively investigated [4,10,40,41]. In aqueous micellar solutions, substrates do not penetrate appreciably into the micelles and reactions occur at or near the surface of the micelles. However, polar substrates and water molecules are believed to be localized in the core of the reverse micelles in non-polar solvents. Many of these studies have used alkylammonium carboxylate surfactants, of general formula $RNH_3^{+}{}^{-}OOCR'$, because they are easy to purify and the alkyl chain lengths can be varied to give homologous series. Another surfactant which

has been widely used is AOT (di[2-ethylhexyl]sulfosuccinate). The dependence of the reactivity in aminolysis of a series of aliphatic p-nitrophenyl esters on alkyl tail length in AOT reverse micelles (acetate fastest, dodecanoate slowest) is the opposite of what one finds in normal micellar systems [42].

In few cases, substantial decrease in rate has been found in AOT reverse micelles. This decrease is attributed to specific microenvironment of the reverse micelles [43]. Several types of organic and inorganic reactions have been investigated in the reverse micellar media of a number of surfactants in different non-polar solvents. Katiyar and coworkers have studied the reactions of triphenylmethane carbocations such as methyl violet, crystal violet, pararosaniline, brilliant green, malachite green etc. with nucleophiles namely OH^-, phenoxide ion and p-cresoxide ion in Igepal CO-530/cyclohexane and Cetyltrimethylammonium bromide/$CHCl_3$-isooctane (1:1) reverse micellar systems. The rate of reactions were found to increase many fold (up to about thousand) than those in bulk water. Interestingly, the rate of reaction is remarkably dependent on the W_o value i.e. $[H_2O]$/[Igepal CO-530]. The rate is highest at the lowest W_o and it decreased with increase in W_o and finally becomes of magnitude similar to that in aqueous medium. The hydrophobicity of the dye molecules and nucleophiles entrapped in micellar system influences the rate of reaction [44]. Investigations of the reactions in these media provide significant information relevant to organic and inorganic reaction mechanisms, small and large

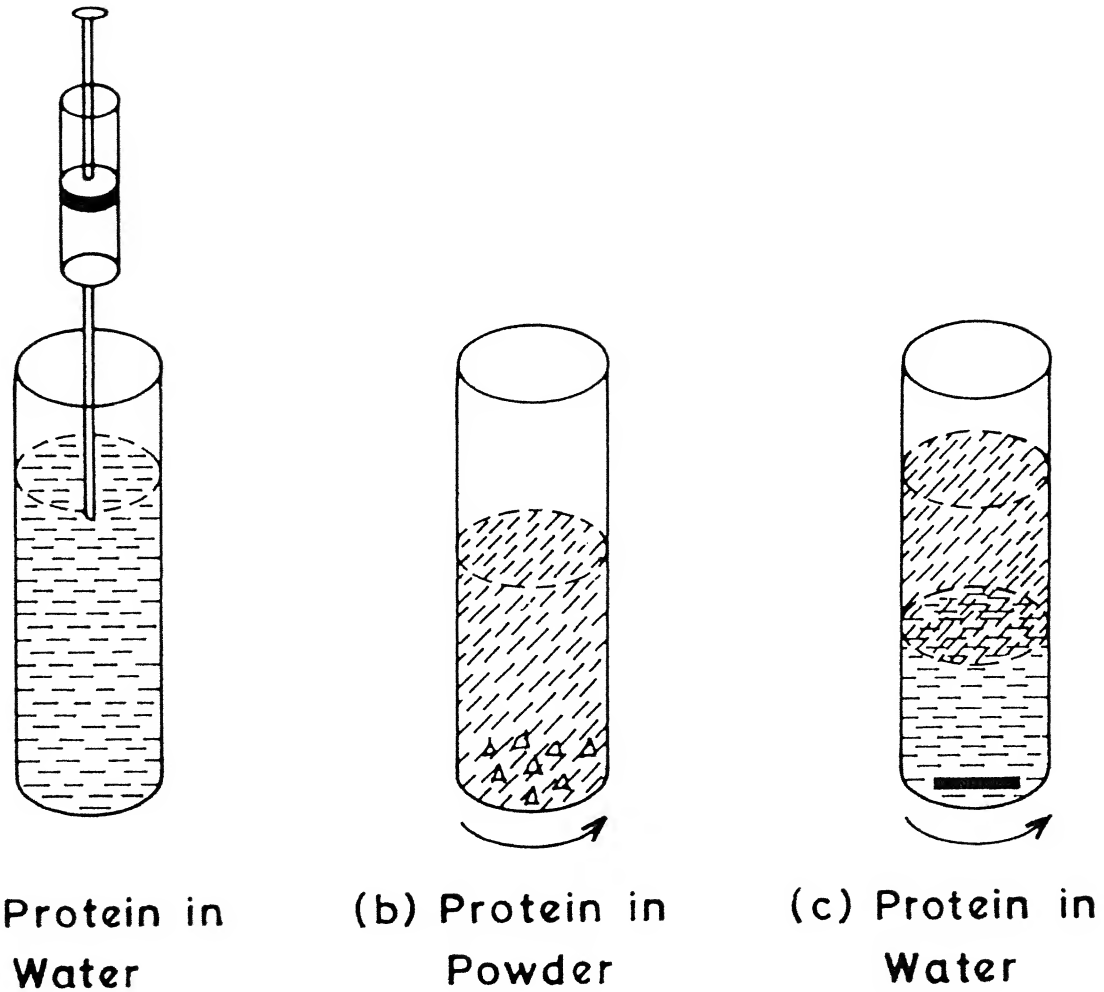

Fig. I.5. The three procedures to solubilize protein in reverse micellar solution. (a) Injection method (b) Extraction from the solid state (c) Phase transfer from aqueous solution.

scale industrial processes, enzymatic and cell membrane interactions [10].

I.5 Solubilization of Proteins in Reverse Micelles

Many biotechnological applications of enzymes and proteins are restricted to aqueous medium. This is because of the reason that most of the proteins are insoluble in organic solvents and are irreversibly denatured when forced into contact with these solvents. But this problem has been circumvented by the novel method of using reverse micelles in organic solvents. Hydrophilic proteins are readily solubilized in hydrocarbon micellar solvents.

Solubilization of proteins is achieved by three different procedures (Fig. I.5). These are as follows.

(a) Injection Method: Complete solubilization of an aqueous protein solution in the reverse micelles.

(b) Solid Extraction Method: Complete or partial solubilization of a dry protein powder in reverse micelles containing water.

(c) Phase Transfer Method in a Biphasic Systems: Complete or partial transfer of proteins from aqueous solution to reverse micellar solution.

In the first procedure suggested by Martinek et al. [45], a small amount of an aqueous protein solution of high concentra-

tion is injected into the reverse micellar solution. The solution becomes optically transparent after gently handshaking or by vortexing for few seconds. The clear solution obtained by solubilization of enzymes in reverse micelles enables the study of enzymes/proteins and other biopolymers under controlled and reproducible conditions [46-50]. Investigations in chapters III & IV will elucidate the usage of this method in detail.

The second procedure i.e. solid extraction method [51] involves the agitation or stirring of dry protein powder in contact with the reverse micellar solution at the desired W_o. High concentrations of protein solutions in reverse micelles can be obtained by adding the solid protein in excess, the portions remaining undissolved after a few hours is removed by centrifugation [52]. This method is particularly useful for the solubilization of lipophilic or membrane bound proteins in reverse micelles. Protein separation can be achieved from the mixture of solid proteins by selective solubilization at optimal conditions. For example, this method has become useful for water insoluble membrane protein like Folch-Pi protein (lipophylin). It has been reported that Myelin basic protein, comprising the myelin structure under the conditions which can be considered similar to those in membrane environment [53,54]. It may be helpful in the study of the characteristics of proteins at liquid solid interface in organic media. Different enzymes/proteins solubilized by this method have been reported in part of chapter II.

The third procedure of protein solubilization in organic olvents i.e. by phase transfer method proposed by Luisi and oworkers [55,56] is shown in Fig. I.5(c) . In this method the rotein is in the water solution and the supernatant is the rganic reverse micellar solution. With gentle mixing either by and or by a mechanical device, and with a rate and efficiency hich depends on buffer, pH and concentration, the protein nitially present in the water phase is transferred into the icellar solution. The process of transfer is relatively slower han that of injection method, may require from minutes to few ours depending on conditions and the nature of proteins 52,56,57]. The protein solubilized in the hydrocarbon phase can e transferred again into the aqueous solution with reverse reatment by changing the nature and concentration of salt in the vater phase. This process is called "backward transfer" and the wo processes combined together are called "double transfer" [52,57].

The advantage of the phase-transfer solubilization method in comparison to other techniques is that the micellar solution is thermodynamically stable and no supersaturation effect is possible. The liquid-liquid phase transfer method is of immense importance and seems to have future in biotechnology for the extraction of proteins. However due to the longer time requirement for the process, the activity of the enzymes may decrease, though not drastically. This method has been utilized by Dekker et al. [58] and Hatton et al. [59,60] for the recovery of enzyme

from the fermentation broth and for protein separations from different mixtures in aqueous solution. We have studied the transfer of many proteins from aqueous solution to organic solution and vice versa. This method has been exploited for the separation of proteins by the reverse micellar organic phase. Part of the chapter II reports these investigations.

I.5.1 Characterization of Protein Containing Reverse Micelles

The solubilization of enzymes/proteins in reverse micelles, in turn, raises many interesting questions. What is the structure of these protein containing micelles ? Where in the waterpool, does the protein reside ? How can we say that the protein goes inside the waterpool or remains outside. How does this depend upon its hydrophilicity and net charge ? Indeed there may be many more questions pertinent to the proteins containing reverse micelles.

On the evidence of the conformation of solubilized biopolymers and activity, the "one-enzyme/protein-per micelle" model, i.e. so called "Water-Shell model" has been proposed for the solubilization of hydrophilic proteins [61-64]. In other words, the biopolymer resides in the waterpool, surrounded by a shell of water which protects it from the surfactant wall and from the hostile hydrocarbon solvent. This situation has been discussed as follows:

If we consider separately the dividing line of low W_o and

high W_o regions, which depends upon the size of the biopolymer, it occurs when the core volume (V_c) of the starting micelles just exceeds the volume of the biopolymer (V_p). This transitional W_o is also called $W_{o,t}$. For small biopolymers (<40,000 dalton), this transitional W_o is below the region where the area per AOT head group reaches its limiting value of 55 $\mathring{A}^2$. If a biopolymer sticks to the surfactant interface at the periphery of the water core then this value of 55 $\mathring{A}^2$ of water surface per AOT head group no longer holds. The various structural possibilities are fully characterized if one can specify for each filled micelle. The best unit here is perhaps the empty micellar core. Above $W_{o,t}$, the biopolymer may enter the waterpool and

1. displace water such that $R_{c,s} = R_{c,f}$, allowing $\bar{n}_{AOT}$ to remain the same, while $W_{o,f} < W_{o,s}$. This requires that $W_{o,e} > W_{o,s}$.

2. swell the core by its own volume so that

$$R_{c,f} = \left(\frac{V_{c,s} + V_p}{(4/3)\pi}\right)^{1/3}$$

$\bar{n}_{AOT}$ will increase, because $R_{c,f} > R_{c,s}$; $W_{o,f}$ will again be less than $W_{o,s}$ and $W_{o,e} > W_{o,s}$.

3. attract additional water, so that $W_{o,f} \geq W_{o,s}$ and $W_{o,e} < W_{o,s}$. This possibility requires both the water and AOT from more than one starting, empty micelle. If two empty micellar cores were to coalesce to form one filled micelle,

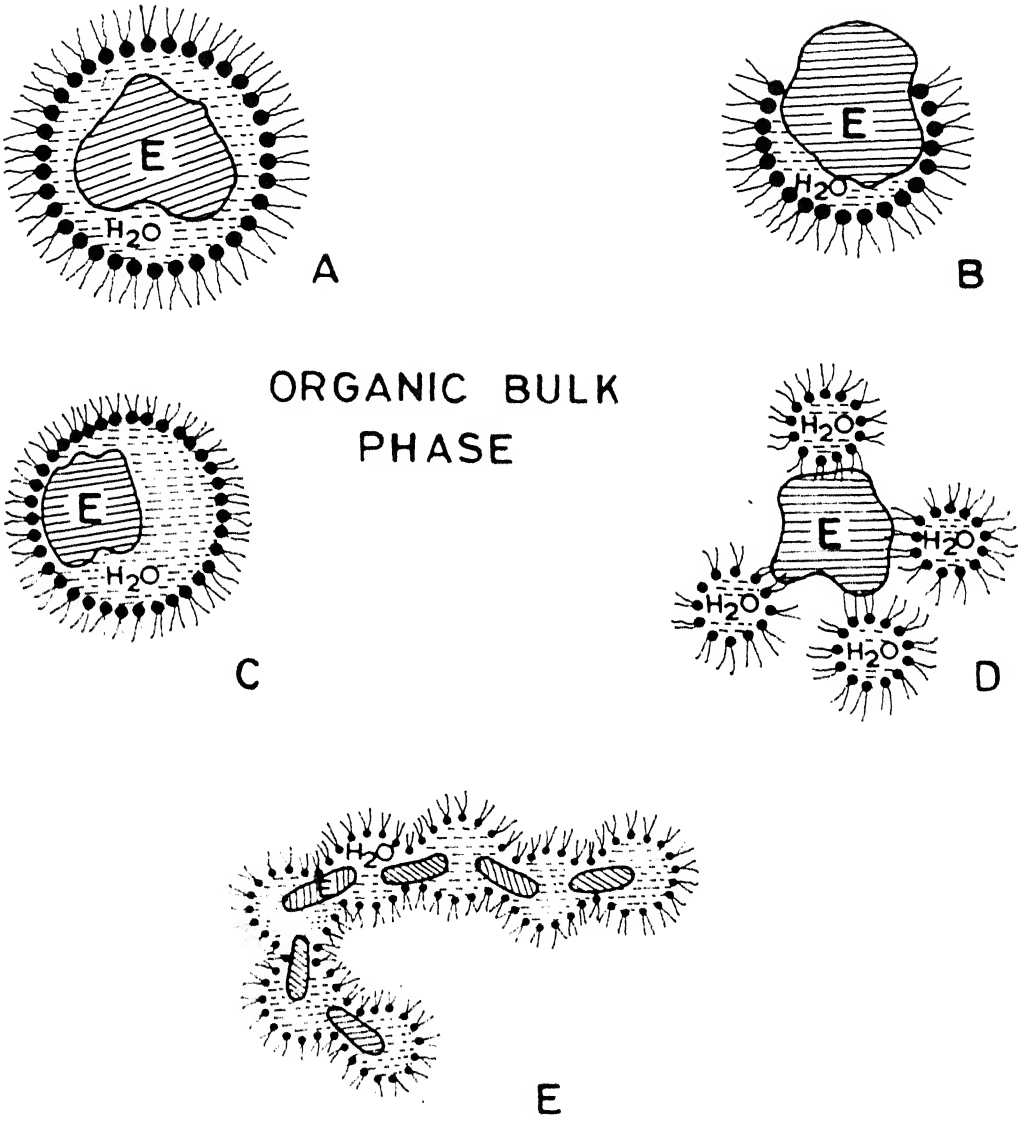

Fig. I.6. Various models for a protein hosted in the reverse micelles. (A) the water-shell model whereby the protein located in the waterpool and separated from the micelle wall by water layers, (B) the case of a protein having a very lipophilic part, (C) protein adsorbed to the micelle wall, (D) protein solubilized by the help of several small micelles, (e) formation of a network among several micelles, bridged by the protein molecules.

the transient dimer model would exist in the kinetics of solubilizate exchange.

Below $W_{o,t}$, the biopolymer cannot fit into the existing micellar core by simply displacement of water, so it must organize a new micelle around itself. Cases (2) and (3) are available; (3) more likely than (2) if a protein of bigger size is to ensure that it retains a water shell.

I.5.2 Structure of Proteins in Reverse Micelles

Despite the accumulation of data on the overall size of protein containing reverse micelles, there is very little information about the internal structure of reverse micelles which host proteins. This section discusses questions like (a) Where are proteins located in the reverse micelles ? (b) Is there a change in conformation and how ? (c) What about the location of the other components in the core of the reverse micelles. Fig. I.6 gives a qualitative representation of the possible situations which may arise [64]. Fig. I.6(a) is the water-shell model whereby the protein is located in the waterpool and is protected from the micellar wall by layers of water. Fig. I.6(b) represents the case of a protein having a very lypophilic part, which tends to interact directly with the hydrocarbon bulk. Fig.I.6(c) is the case of a protein which interacts strongly with the micellar wall. Fig. I.6(d) shows the case of a protein solubilized with the help of several micelles that is via hydrophobic interaction of protein with several micelles. Whereas

Fig. I.6(e) represents a rare case in which there is formation of a fused network of several micelles, bridged by the protein molecules [65].

The water-soluble proteins and other biopolymers prefer to stay in the waterpool of reverse micelles. Many reports support their solubilization by "water-shell model". For example, conformation studies on the solubilization of few proteins like ribonuclease [66], α-chymotrypsin [54], lysozyme [50] etc. in the reverse micelles have been carried out. It was found that circular dichroism spectra of ribonuclease in the aqueous and reverse micellar solutions were identical. For α-chymotrypsin, the same is true in the U.V. region. Fluorescence studies on α-chymotrypsin [54] and lysozyme [50] in AOT/isooctane indicate that the indigenous tryptophan experiences an environment which is more apolar than water, particularly at low W_o.

In most studies on enzymes in reverse micelles, reported by other groups [67,68] and by Katiyar et al. [49,69], the mechanism of enzyme action appears to be the same as in aqueous solution. Structural investigation on cytochrome-C solubilized in AOT/isooctane carried out by Pileni and coworkers [70] gave evidence of the adsorption of protein to the interface of the reverse micelle. The adsorption markedly perturbs the micellar structure, the protein state and reactivity.

I.5.3 Molecular Weight Determination of Protein Containing Reverse Micelles

Sedimentation analysis has been one of the most informative and widely used method for the molecular weight determination of macromolecules [71,72]. The following equation has been used for solubilized proteins.

$$S = (D/RT)M(1- \bar{v}\rho) \quad .. (1)$$

Where S is the sedimentation coefficient of a particle with molecular weight M and the partial specific volume $\bar{v}$ in the solvent with density ρ . D is the diffusion coefficient, R is the gas constant, and T is the absolute temperature. When the concentration of reverse micellar solubilized protein is not too high it can be assumed that the stoichiometric ratio is one protein molecule per reverse micelle. Further it has been considered that entrapment of a protein molecule into a reverse micelle causes redistribution of free and bound water. Under these conditions, following relations are fulfilled:

$$D_f \simeq D_o \quad .. (2)$$

$$v_f \simeq (M_o/M_f)\bar{v}_o \quad .. (3)$$

$$M_f = M_o + M_p \quad .. (4)$$

Where f and o denote the parameters for filled and unfilled micelles and M_p is the protein molecular weight. From relation (1) - (4), we get

$$M_p = M_o\{(S_f/S_o)-1\}(1- \bar{v}_o\rho) \quad .. (5)$$

Where M_o, v_o and ρ are well known characteristics of the empty reverse micelle which do not depend on the nature of the protein. Finally determination of the ratio of sedimentation coefficients (S_f/S_o) gives the molecular weight of protein α-chymotrypsin [71]. It has been reported that the diameter of inner cavity of reverse micelles at W_o = 12, is about 45 Å. This value corresponds well with the α-chymotrypsin molecular size (40 x 40 x 50 Å) [73]. Bonner et al. [62] have determined the molecular weight of protein containing reverse micelles of AOT/isooctane, e.g. for lysozyme at W_o = 22.5, M_r = 2,670,000 dalton. Bonner et al. and Levashov et al. [62,71] provide the different data of protein containing micellar parameter in the case of proteins, ribonuclease, lysozyme, horse liver alcohol dehydrogenase in tabular form.

I.6 Extraction of Proteins Using Reverse Micelles

This is based on the principle that proteins can be transferred selectively from an aqueous phase into a reverse micellar phase and vice versa. A considerable amount of work done in reverse micelles recently, allows one to utilize reverse micellar system as a possible tool for bioseparations (i.e. separation of biopolymers from their native milieu and from other undesirable species.

I.6.1 Factors Affecting Protein Solubilization

Much of the ongoing research in the area of protein

separations by reverse micelles has centered around the partitioning behavior of various proteins between aqueous phase and reverse micellar phase and on the effect of various parameters on the solubilization of proteins in reverse micelles [52,57,58,74].

A number of parameters affect the solubilization of protein in reverse micellar solution. These parameters are: surfactant structure, and concentration, pH and ionic strength of the aqueous phase, micelle size (which is affected by water content & solvents used), temperature, protein charge, solvent structure, phase ratio and additives (like ions & ligand that binds the protein favorably). These system characteristics, not only affect the equilibrium concentrations, they also govern the rates of transfer into and out of the reverse micelles.

I.6.1.1 Effect of pH

The pH of the solution may affect the solubilization characteristics of a protein primarily in the same way in which it modified the charge distribution over the protein surface. In addition to this, change in conformation of protein with the change in pH may also play a significant role in this context. For example, Goklen & Hatton [57,59] had observed the solubilization behavior of several proteins like cytochrome-C, ribonuclease and lysozyme in AOT/isooctane. There was a sharp transition in solubilization behavior at the pI of each protein, which demarcated the region of no solubilization (pH > pI) from

that of almost quantitative transfer to the organic solution (pH < pI). In the case of cationic reverse micelles trioctylmethylammonium chloride (TOMAC)/cyclohexane extraction of trypsin, α-chymotrypsin and pepsin showed little dependence on the protein pI [56].

I.6.1.2 Surfactant Concentration Effect

The intake of proteins in the reverse micellar solution is increased by increasing the surfactant concentration. Hatton et al. [75] observed the broadening of pH solubilization peak with increase in surfactant concentration. Woll and Hatton [76] investigated the equilibrium solubilization characteristics for the two proteins ribonuclease and concanavalin-A at different AOT concentrations over a range of pH values below the pI's of the proteins. These authors have attributed the phenomenon "the increase in solubilization with increasing surfactant concentration" in terms of phenomenological thermodynamic model based on mass action kinetics.

I.6.2 Product Recovery & Activity

The efficacy of reverse micellar extraction for the large scale recovery of proteins depends on the ease with which the protein can be stripped from the loaded organic phase, and the extent to which enzyme activity or biological function is retained by the recovered product. Todate few studies have been done, and it has been found that different proteins behave

lifferently in backward extraction, depend on properties such as)H and ionic strength. Few proteins are completely stripped out :rom reverse micellar phase while others get partially :ransferred to aqueous solution. Hatton and coworkers [77] have .nvestigated the complete forward and backward extraction for .ysozyme and ribonuclease etc. Whereas in the work of Woll et ıl. [76], the proteins ribonuclease-A and concanavalin-A could ıot be recovered using pH and ionic strength changes, indicating :hereby a strong protein-surfactant interaction in these cases. 'his problem was resolved by the addition of 10 to 20% (v/v) :thyl acetate mixed with the AOT/isooctane solution. In this :ondition complete recovery of both concanavalin-A and 'ibonuclease-A could be obtained with no apparent loss in .ctivity. The retention of biological activity during the :xtraction/stripping cycle and the reuse of the micellar solution 'or multi extraction steps are two important considerations in .he large scale application of reverse micellar extraction)rocesses.

.6.3 Protein Separations

Very few studies on the separation of proteins, with both ynthetic protein mixtures and real fermentation media have been eported. Goklen and Hatton [59] investigated the separation of rotein mixtures of cytochrome-C, lysozyme & ribonuclease-A. 'oll and Hatton [76] investigated the separation of binary ixture of ribonuclease-A and concanavalin-A. In extension of

their work to the affinity partitioning model, they observed that the selectivity for one protein over the other via protein-specific interactions with appropriate affinity surfactants could be predicted based on the single protein extraction studies [77]. Rahaman and coworkers [78] have achieved the selective extraction of proteins from real fermentation media. They have recovered and purified an extracellular alkaline protease (a detergent enzyme) from an untreated fermentation broth. The authors have found that by increasing the aqueous/organic volume ratio, the mass recovered per unit volume of extractant changed little, but the amount of active component extracted increased about 4-fold under the same experimental conditions.

I.6.4 Direct Recovery of Intracellular Enzymes

An interesting approach for the recovery of intracellular enzymes has been proposed by Giovenco et al. [79], who injected a suspension of whole cells directly into a CTAB/hexanol-octane (1:9) reverse micellar solution. The enzymes are released from the cell by disintegration of cell membranes by surfactant. They suggested that salt detergent matching, with pH as an extra degree of freedom, is an important factor in the recovery of active enzyme. Besides this, W_o was reported to be a selection criterion in the recovery of desired proteins. There is enough scope for the optimization of this process to obtain greater purification factors and higher yield.

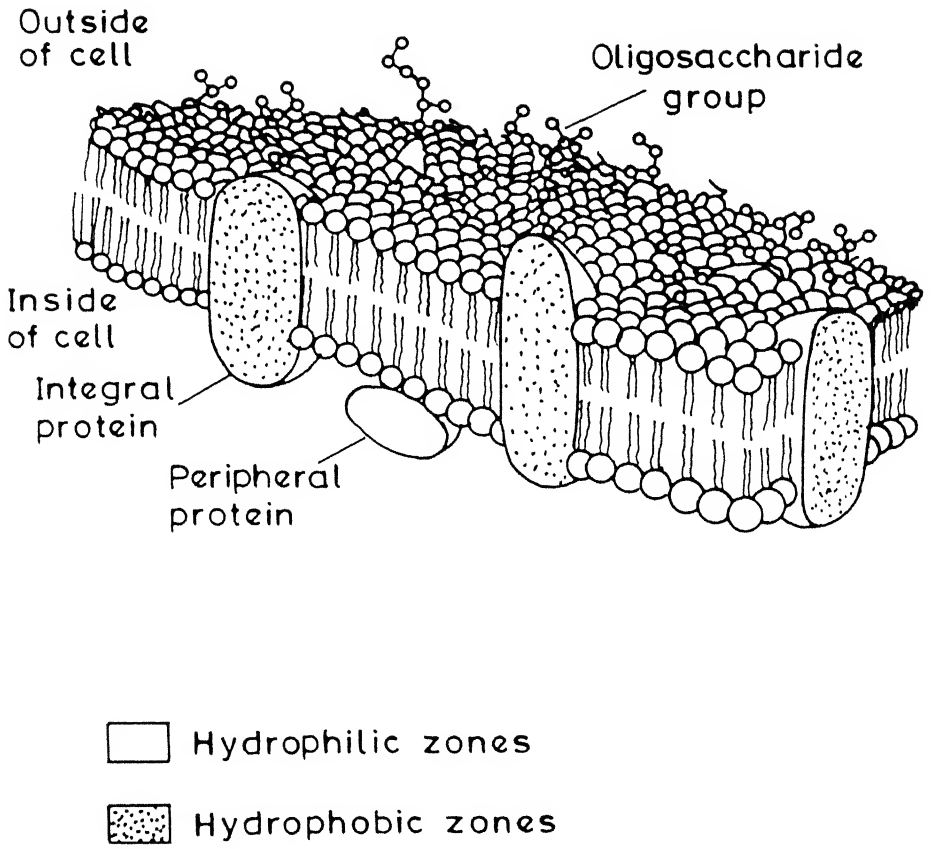

Fig. I.7. The Singer-Nicolson fluid - mosaic model of membrane structure.

I.7 Enzymology in Reverse Micelles

Enzymology in reverse micelles is a novel approach to problems of molecular biology [80,81]. In living cells, enzymes mostly act either on or near the 'water/organic medium' interface that is on the surface of biological membranes or inside them, or in mobile complexes with macromolecular components of the cell, [82]. The study of enzyme reaction in reverse micelles provides an approach to investigate the in vivo action of enzymes in in vitro studies which usually are carried out in aqueous buffer [83].

It is well known that the flat bilayer of lipid molecules constitute the main structural pattern of biological membranes. However, the notion of the lipid bilayer, the most possible way of organization of membrane lipids, which represents the special feature of widely accepted fluid mosaic model [84] of biological membranes as shown in Fig. I.7 does not agree with established facts of structural rearrangements of lipids, viz. from the bilayer to the hexagonal phase [85]. Further investigations of the structure of lipid membranes [86] resulted in the discovery of other types of non-bilayer lipid structure, in particular, so called lipidic particles, representing 'reverse micelles' sandwiched between monolayers of the lipid bilayer. Taken together, the above data allow one to conclude that model studies of enzymatic catalysis in microheterogeneous media, like the systems of hydrated reversed micelles of surfactants [80,87] are of great importance in understanding the enzyme function in

TABLE I.2: Various Reverse Micellar Systems in which different enzymes have been studied in Organic Solvents

SURFACTANT	SOLVENT	SURFACTANT	SOLVENT
Anionic		**Cationic**	
AOT Sodium(bis,[2-ethylhexyl] sulfosuccinate $ROCCH_2CHCOR$; R=2-ethylhexyl $SO_3^-Na^+$	Isooctane , n-Octane, Heptane, Cyclohexane, Heptane mixed with phospholipids	DAP Dodecylammonium propinate $CH_3(CH_2)_{11}\overset{+}{N}H_3\ \overset{-}{O}-\overset{O}{\overset{\|}{C}}-CH_2CH_3$	Cyclohexane
		TOMAC Trioctylmethylammonium chloride	Cyclohexane
SDS Sodium dodecylsulfate $CH_3(CH_2)_{11}SO_4^-Na^+$	Cyclohexane/n-hexanol (2-5 %. water)	**Nonionic**	
		Brij - 56 Polyoxyethylene(10)-cetylether $C_{16}H_{33}(OCH_2CH_2)_{10}OH$	Isooctane, Heptane, Hydrocarbon/ n-Hexanol, Cyclohexane, Octane
Cationic			
CTAB Cetyltrimethylammonium bromide $CH_3(CH_2)_{15}\overset{+}{N}(CH_3)_3Br^-$	n-Octane/$CHCl_3$(1:1) Octane, Benzene, Hexane, Decane, Dodecane/n-Hexanol(1:1) Isooctane/$CHCl_3$(1:1) Isooctane/Butanol, Heptane/$CHCl_3$(1:1)	Brij - 96 Polyoxyethylene(10)-oleylether $C_{18}H_{35}(OCH_2CH_2)_{10}OH$	Cyclohexane, Octane
		Triton X-100	Cyclohexane/ n-Hexanol (1-13 %. water)
TDTAB Tetradecyltrimethylammonium bromide $CH_3(CH_2)_{13}\overset{+}{N}(CH_3)_3Br^-$	Isooctane/$CHCl_3$(1:1)	**Zwiterionic**	
		Lecithin(Phosphatidyl cholines CH_2OCOR CH_2OCOR_2 $CH_2OP(O)O(CH_2)_2\overset{+}{N}(CH_3)_3$	Ether/CH_3OH(5 %.) Decane, Hexane
Cetrimide Mixed alkyltrimethylammonium bromide	Isooctane/$CHCl_3$(1:1)		

natural lipid systems.

I.7.1 Enzyme Catalysis in Reverse Micelles

Many enzymes have been solubilized and studied in reverse micellar solutions. It is not surprising that lipolytic enzymes retain their enzymatic function in such microheterogeneous media [88,89]. Normal functioning of hydrophilic enzymes in this media has made reverse micellar system a viable alternate media for the study of enzymes. The first report of solubilization of hydrophilic protein α-chymotrypsin in trioctylmethylammonium chloride/cyclohexane was reported by Luisi et al. [55]. Later on Martinek et al. [90] and Menger [91] showed the activity of α-chymotrypsin in hydrocarbon using AOT. Table I.2 lists some reverse micellar systems in which enzymes have been solubilized and studied in organic solvents. The activity of enzymes in reverse micellar system is remarkably dependent on W_o, pH, surfactant concentration etc.

I.7.1.1 Effect of W_o

One of the striking effects observed in the study of enzymes in reverse micelles is the dependence of catalytic activity of the solubilized enzymes on W_o. This parameter determines the size and properties of the micromedium within the inner cavities of the reverse micelles. The W_o dependence of the catalytic activity has been observed for enzymes such as α-chymotrypsin

[47], cholesterol oxidase [92], lysozyme [50], phospholipase A_2 [89], pancreatic lipase [93], dihydrofolate reductase [94], malate dehydrogenase [49] and yeast alcohol dehydrogenase [95] etc. Normally, this dependence is bell shaped. The activity is maximum at an optimum value of W_o which is different for different enzymes.

I.7.1.2 Effect of pH

The rates of enzyme catalyzed reactions and associated kinetic parameters depend on pH of the waterpool inside the reverse micelles. After the transfer of enzyme and substrate solutions from aqueous to reverse micelles a significant shift occurs in the optimum pH for maximum enzyme activity. This phenomenon can be attributed to different reasons. For example (a) the use of inorganic surfactants forming a charged layer (electrical double layer) around the enzyme molecule, may lead to a local shift of pH. The apparent shift of pH (and correspondingly of the effective pK_a in this case is usually 1-2 pH units [49,50,54,56,74,75,92-96,102-106]. (b) the acid base properties of ionogenic groups of the enzymes solubilized in the reverse micelles can vary with their microenvironment. (c) the possibility of a conformational change in the enzyme on solubilization can also alter the observed pK_a of its ionogenic groups, including the functional groups involved in the enzyme reaction.

I.7.1.3 Substrate Specificity

Martinek et al. [97] have studied the specificity of horse liver alcohol dehydrogenase solubilized in AOT/n-octane. The enzyme catalyses the oxidation of alkanols to the corresponding aldehydes as shown in scheme I.1. The catalytic activity was found to depend on the carbon chain length of the substrate. The substrate specificity changed in as much as octanol was the best substrate in water whereas in reverse micellar solution butanol became the better substrate. This phenomenon was explained by assuming that more hydrophobic substrates are extracted to a higher degree in the organic solvent and their local concentration near the active center is therefore lower.

$$H(CH_2)_nOH + NAD^+ \overset{LADH}{\rightleftharpoons} H(CH_2)_{n-1}CHO + NADH + H^+$$

Scheme I.1

I.7.1.4 Superactivity

Superactivity of enzymes entrapped in reverse micelles is one of the most interesting observation. Few enzymes due to numerous factors, which either compensate or supplement each other, show this rare phenomenon of increased activity in comparison to the optimum activity in water. The enzymes which show high superactivity are α-chymotrypsin [47], laccase [98], acid phosphatase [99] and peroxidase [100] etc. Other enzymes which display superactivity in the micellar media are dihydrofolate reductase [94], yeast alcohol dehydrogenase [95]

and glucose 6-phosphate dehydrogenase [101] studied by Katiyar and coworkers.

Regarding the mechanism of superactivity, it is known that under optimum conditions, when the degree of hydration W_o is optimum, the inner diameter of the empty micelles practically corresponds to the size of the entrapped enzyme molecule. If the enzyme maintains the most viable conformation at the optimum W_o then it may result in superactivity. There is a good coincidence in the radii of the different protein molecules (r_p) and corresponding inner cavities of the optimum micelles (r_m) [102]. The second important evidence came from the study of ESR of spin label introduced into the active centre of solubilized α-chymotrypsin [103]. In this experiment, the value of minimum rotation speed of the spin label in the active centre of the enzyme coincides with the optimum of its catalytic activity.

Above observations favor the hypothesis that the superactivity may be due to the relatively high rigidity of the surfactant shell surrounding the molecule of the solubilized enzyme.

I.8 Application of Reverse Micelles in Various Areas

Many fascinating applications of enzyme containing reverse micelles have been reported over the last few years as the conditions for the activity and stability of enzymes in reverse micellar systems have been worked out. The field of enzyme

catalyzed organic synthesis in reverse micelles is in its infancy nevertheless, shows promise for implementation on an industrial scale. Due to specific requirement of one enantiomer, an enantiomeric resolution of amino acids has been reported. Recently, immobilized acylase has been used to hydrolyze only the L-isomer of acyl D,L-amino acid mixtures [104]. With reverse micelles, proteases could be used to selectively esterify only the L-isomer of an amino acid, permitting resolution to become a separation of an acid from an ester [105]. The other use of reverse micelles is in the selective modification of ester bonds of triglycerides or diesters. The abundance of oils and fats available in the biosphere can often be modified for use as chemical intermediates in the food and consumer product industries. Enzymes in organic solvents function as selective catalysts for trans-esterification and hydrolysis reactions, whereas in aqueous systems, nonspecific reactions can occur [106-107].

A multitude of steroids are pharmacologically active, many of which can be obtained by modification of a few precursor steroids to a variety of active compounds. Such modifications are difficult to carry out with traditional synthesis, because of the large number of potential reaction sites in a steroid molecule. Site specific steroid conversions by enzymes are preferably carried out using organic solvents because of low steroid solubilities in water. Laane and coworkers [108-109] have reported the solubilization of the enzyme (viz. hydrogenase,

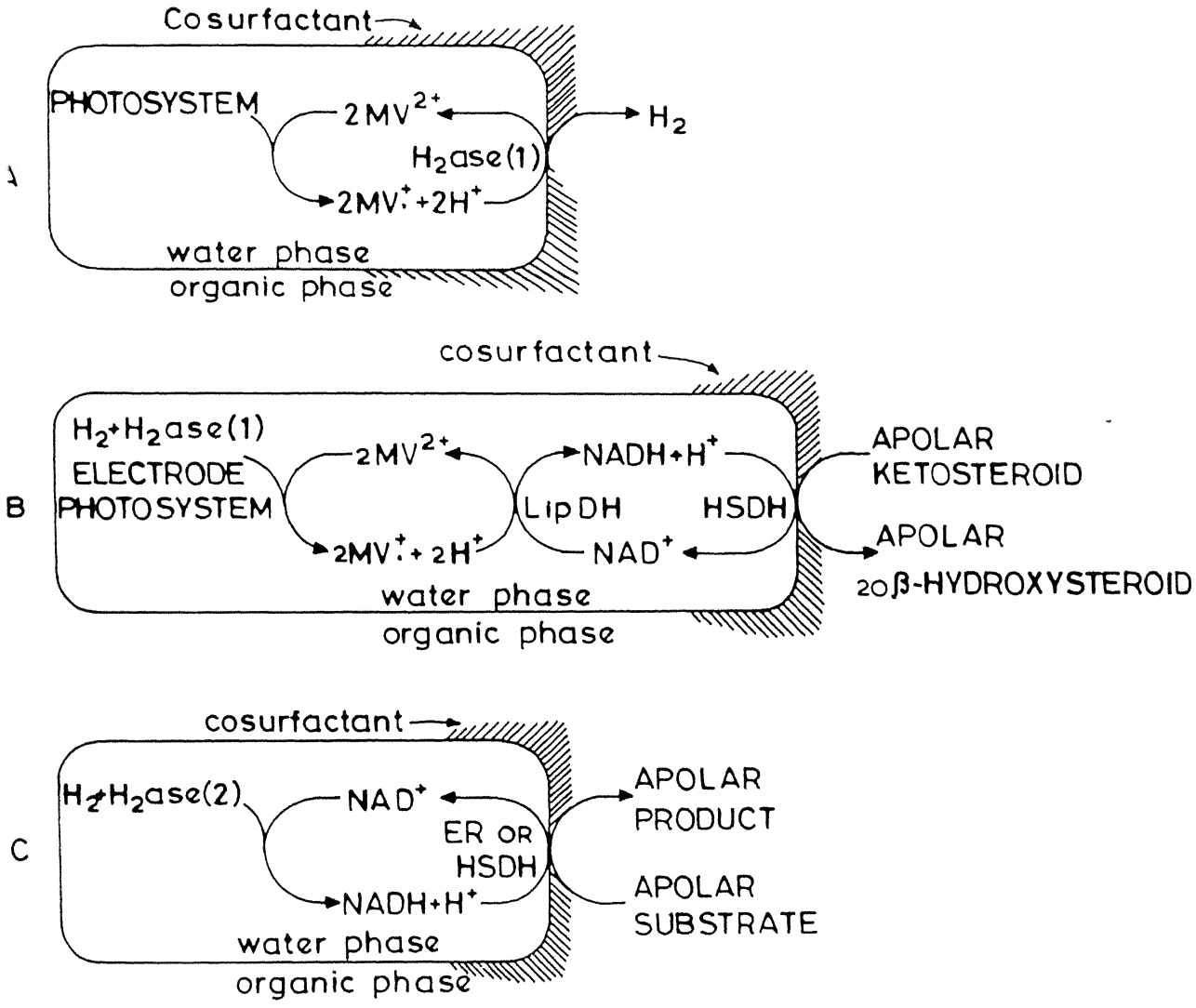

Fig. I.8. Schematic representation of (A) photosensitized production of hydrogen by hydrogenase (B) Indirect NADH-mediated reduction of 20-ketosteroids, (C) Direct NADH-mediated reduction of enolates and 20-ketosteroids.

lipoamide dehydrogenase and steroid dehydrogenase) to specifically reduce a water insoluble ketosteroid to its corresponding hydroxy form using gaseous hydrogen or the electricity as the reducing species (Fig. I.8). The enzymes were found to be more stable in reverse micelles than in aqueous solution and the product could be isolated batchwise by transferring back from reverse micellar medium to aqueous medium followed by the precipitation of the surfactants and evaporation of organic solvents.

Oligopeptides are being manufactured for a number of applications including artificial sweeteners, pesticides, and pharmaceuticals. However, current synthetic methods are small volume batch processes. Enzymes solubilized in organic solvents may provide the large scale, more efficient processes needed for the cost effective synthesis of these peptides. Luisi and coworkers [110] have used reverse micelles for the enzymatic reactions involving lipophilic reagents. For example, a hydrocarbon soluble tripeptide has been synthesized using α-chymotrypsin enzyme.

$$\underset{(A)}{\text{Z-Ala-Phe-OMe}} + \underset{(B)}{\text{H-Leu-NH}_2} \rightleftharpoons \underset{(C)}{\text{Z-Ala-Phe-Leu-NH}_2} + \text{MeOH}$$

Scheme I.2

Z represents the benzyloxycarbonyl protecting group. Both A and C are practically insoluble in water but soluble in isooctane. Luisi and coworkers have utilized a hollow fiber reactor for

these peptide syntheses.

A reverse micellar system containing Tween 85 and water in isopropylpalmitate has been able to solubilize whole cells of E. Coli and of Acinetobacter Calcoaceticus [111]. The cells remained viable for at least one day and retained enzymatic activity (e.g. β-galactosidase activity in E. Coli) for an even longer period of time. Considering the use of the cell containing micellar system, the solubilization of A. Calcoaceticus is an interesting example, because these cells are able to degrade alkanes which can be added as a co-solvent to isopropyl-palmitate.

Often the enzymes denature or lose their activity at very low temperature. Reverse micelles can provide a possible milieu for studying enzyme mediated processes at sub-zero temperature. This in turn, considerably slows down reactions and thus allows the leisurely examination of the kinetics of formation and decomposition of short lived intermediates. The reverse micelles stabilize supercooled water droplets inside the micellar core against freezing due to heterogeneous nucleation. Douzou and coworkers [112,113] have successfully studied the behavior and activity of cytochrome-C and trypsin in this system at cryogenic state.

In a recent report, it was reported that the antibodies have been found to be stable and show immunoactivity in the micellar media. The alterations in the catalytic activity of the enzyme peroxidase after its interaction with antibodies against the same

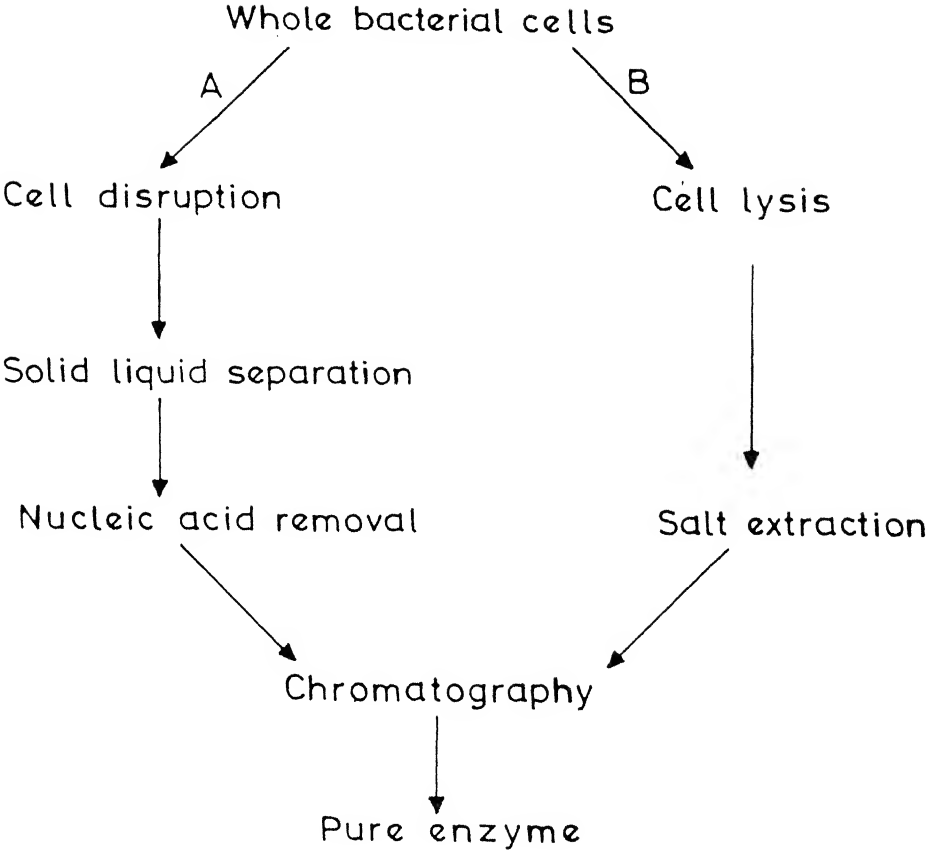

Fig. I.9. Experimental procedure of enzyme purification from whole bacterial cells using reverse micelles.

antigen have been studied in the reverse micellar system of AOT and Triton X-45 in heptane [114]. The change of various parameters like pH, W_o, surfactant concentration etc. remarkably affected the interaction between the enzyme and antibody. In another report by Durfor et al. [115] specially generated monoclonal antibodies work as enzymes in reverse micelles.

A new, rapid pre-chromatography isolation procedure for intracellular enzymes from whole bacterial cells has been developed using reverse micelles [79]. The method involves two relatively simple steps as shown in Fig. I.9. In the first step, bacterial cells are disintegrated by the surfactants in the reverse micellar medium, and in the second step the liberated enzymes are extracted from the micellar phase into an aqueous phase. Giovenco et al. have extracted and purified the three enzymes namely β-hydroxybutyrate dehydrogenase, isocitrate dehydrogenase and glucose 6-phosphate dehydrogenase from the bacterial cells, A. vinelandii using CTAB/hexanol-octane (1:9) reverse micellar system.

The use of hardened micelles for the intra or transcellular transport of drugs to specific cells in organs is a recent approach used to specifically target cells with drugs. For this purpose, the drug containing hardened micelle is transported through the cell wall with the aid of endocytosis processes [116]. This target has been shown with an antitumor drug, Actinomylin D. etc. [117]. There is a growing interest in using reverse micellar systems (or water-in-oil microemulsions) in

smetics and pharmacological formulations. The aim is to rmulate an oil phase in which water soluble components are nogeneously dispersed and still active. Haering et al. have rmulated that the organic solutions containing reverse micelles n be thickened and even transformed into gels by gelatin trapped in the waterpools [118]. In this way one could prepare e solution of a biocompatible oil, or its gel which contains tive hydrophilic enzymes, or hormones, water soluble vitamins, .c. These solutions can be utilized for topical preparations or ssibly for transdermal transport (the latter is particularly ıder study in several research groups in industry).

.9 Objective of the Present Work

Reverse micelles hold the promise of several fascinating pplications in biotechnology. There has always been a need for he successful, continuous, economical and large scale bioprocess echnology for concentrating and separating the desired nzyme/protein product or other bioproducts from complex ermentation broths and cell culture media. Genetic engineering nd recombinant DNA techniques have progressed tremendously in reating a large variety of products. Nevertheless, the successful commercialization of these products suffers due to .ack of efficient methods for continuous large-scale recovery of proteins from fermentation cell culture media.

Our aim in investigating the solubilization of different proteins of diverse characteristics (viz. in complexity, size,

molecular weight, sub-units etc.), was to find out the optimum conditions under which single proteins can be solubilized selectively in the reverse micellar system, namely AOT/isooctane and then complete back transfer of proteins from micellar to aqueous phase. Depending on the different steps of experimental procedures and solubilization behavior of proteins under different conditions like pH, ionic strength of the aqueous phase, a strategy needs to be developed. This approach has the potential for the development of procedures for the continuous and large scale extraction and recovery of proteins and other bioproducts from complex mixtures from natural broth etc.

The next thrust of the work was to examine the behavior of few important enzymes in non-polar reverse micelles. Unfortunately, the enzymes being soluble in water and active in the particular conformation, do not tolerate the direct contact with non-polar solvents. They often lose their activity and the substrate specificity in this media. At present, enzymes used as catalysts in the reverse micelles in organic solvents seem to hold promise for the synthesis of many important compounds at high rate and desired specificity, which are normally not feasible in water.

Todate, most of the enzymes studied in reverse micelles by few groups, are of low molecular weight and lesser sub unit (usually monomer/dimer). With the increase in complexity of enzyme structure, significant change in behavior of enzymes in reverse micelles have been observed. It is proposed to study

three enzymes which are reasonably bigger in size and diverse in complexity. For example, the flavo enzyme glutathione reductase, is a dimer with one FAD per monomer of molecular weight 50,000 dalton. On the other hand, lactate dehydrogenase and pyruvate kinase are oligomeric. The detailed study of kinetic characteristics of these enzymes can demonstrate the versatility of reverse micellar media for carrying out enzyme catalyzed reactions.

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CHAPTER II

EXTRACTION AND SEPARATION OF PROTEINS USING REVERSE MICELLES

II.1 Introduction

Though the fermentation aspects of traditional as well as genetically manipulated microbes are well developed in biotechnology, recovery and purification of bioproducts have not received due attention. There have been very few industrially important innovations in bioseparation. Novel bioseparation techniques that are efficient and can be scaled up are needed to fulfil the promise of biotechnology. The traditional methods for the recovery of biomolecules from complex mixtures, such as chromatography and electrophoresis are limited to analytical or preparative scale.

In recent years, a new impetus to the search of efficient methods for the separation of proteins was given by the discovery that liquid-liquid extraction can be successfully used for recovery of proteins and other biopolymers. This unit operation started being considered seriously as a viable approach for bioproduct separations and purifications, primarily as a result of studies of Kula and coworkers in biphasic aqueous polymer

system [1,2] at pilot scale. In this system each phase is primarily aqueous. The production of separated aqueous phases is achieved by dissolution of two incompatible polymers, such as polyethylene glycol (PEG) and dextran. The resulting phases have greater than 75% of water and either dextran or PEG rich. It has also been demonstrated that PEG rich phase may have almost no dextran. A summary of eight enzymes purified from microorganisms in a PEG-Dextran system has been given in [3]. The drawback of this system is the high cost of dextran which can not be used continuously. In this process the polymer phases may be separated by decanting or centrifugation, and then stripped of desired product giving polymer recovery.

The use of aqueous-organic two phase system is in practice for antibiotic recovery [4]. Many important antibiotics like penicillin, erythromycin and bacitracin etc. have been concentrated and purified via aqueous to organic and again back to aqueous from organic phase. Unfortunately this organic-aqueous two phase system can not be employed for the bioproducts like proteins, enzymes and nucleic acids etc. This is because most of the proteins are insoluble in organic solvents or are irreversibly denatured when forced into contact with these solvents.

The need for an extracting phase which does not harm labile enzymes and other proteins, which can be conveniently used even for large scale product recovery, and which allows purification of modesty stable whole organelles and other substructures, is

xtremely important. But this problem was circumvented by the ovel method of using surfactants in solvents which formed reverse micelles' (cf. chapter I). Extraction of nzymes/proteins by organic solvents using reverse micelles is a elatively new concept. The reverse micelles are able to host roteins/biopolymers in an aqueous environment, effectively hielding them from the organic solvent molecules in which they re solubilized [5-10]. They are able to solubilize the iopolymers selectively from the mixture of different roteins/enzymes and other biomolecules depending on the arameters like pH, ionic strength of the aqueous solution and haracteristics of surfactants and solvents of reverse micellar ystem.

The first report on the transfer of proteins from an aqueous hase to a reverse micellar phase and vice versa was from Luisi t al. [11]. This work was further extended recently by Hatton t al. [12-15] and by groups in Wageningen [16-17]. Thus selective and controlled solubilization of proteins and other biopolymers in reverse micellar organic phase is a promising technique that has the potential to be developed into a liquid-liquid extraction technology for bioseparation. In this chapter the solubilization behavior of several proteins/biomolecules into reverse micelles was examined to establish whether this process can be utilized for the separation and extraction of proteins. For this purpose, two techniques of solubilization have been used (i) the direct extraction of the enzymes/proteins powder into

he reverse micellar solution and (ii) the phase transfer of roteins from an aqueous solution into a supernatant micellar olution. Influence of structural parameters of the proteins, as ell as the influence of external parameters (pH, salt oncentration) on the specificity of the solubilization process ere examined.

II.2 Experimental Section

II.2.1 Materials

Lipase from Candida Cylindraccae, Type VII; Creatinephospho Kinase from rabbit muscle, Type I; Baker yeast alcohol dehydrogenase; porcine heart cytochrome-C reductase; cytochrome-C from horse heart, Type III; Bovine Serum Albumin (BSA), 98-99% pure; Nicotinamide adenine dinucleotide, reduced (NADH) from yeast, grade III, disodium salt and lipoic acid (or DL-6,8-Thioctic acid) were obtained from Sigma Chemical Co. St. Louis, U.S.A. Pepsin from porcine stomach, was a product from Worthington, Millipore Corporation, New Jersey, U.S.A.

Dioctyl sodium sulfosuccinate, AOT (Aerosol-OT), 100% was procured from American Cyanamid Co. Wayne, N.J., U.S.A. and Sargent Welch Scientific Co., Illinois, U.S.A. AOT was purified by the method reported in the literature [18]. It was observed that the U.V.-Vis absorption spectra of AOT from both sources had no peak in the u.v. range 240-300 nm. The purified AOT were dried over P_2O_5 in an evacuated desiccator for several hours just prior to use. Cetyltrimethylammonium bromide (CTAB) was used from Sigma

Chemical Co. U.S.A. This surfactant powder was purified by the reported procedure [19] and also kept over P_2O_5 in a vacuum desiccator before use. Isooctane, purum grade was purchased from Fluka, Switzerland and chloroform, HPLC and spectroscopy grade from S.D. Fine Chemicals Pvt. Ltd., Bombay, India.

Buffer components potassium phosphates, trizma base, glycine, etc. were obtained from Sigma Chemical Co. U.S.A. Potassium chloride, $MgCl_2$, potassium hydroxide, HCl etc. all were AR grade from Merck, India. Folin-Ciocalteau reagent was purchased from Sigma Chemical Co., U.S.A. All other chemicals were of analytical grade.

II.2.2 Methods

II.2.2.1 Reverse Micellar Solution

The reverse micellar solution of anionic surfactant, AOT and cationic surfactant CTAB were prepared by dissolving the required amount of these surfactants in isooctane and in the mixture of chloroform-isooctane (1:1, v/v) respectively.

II.2.2.2 Protein Solubilization by Solid Extraction Method

All extraction experiments from the solid state were carried out in 10 ml tightly stoppered glass vials. Each vial contained 2 mg of enzymes/proteins powder in dry form to which 2 ml of a 0.1M AOT/isooctane solution or 0.1M CTAB/$CHCl_3$-isooctane (1:1, v/v) solution, having known water content (expressed in terms of

W_o = $[H_2O]/[Surfactant]$), with preadded water were kept in separate sets. Each set was vortexed for 15 minutes and then left to stand for ~20 hours at ~30°C. The undissolved material was separated by centrifugation. The protein concentration in the reverse micellar solution was determined spectrophotometrically at 280 nm assuming the same extinction coefficients as in water, and by Lowry method for undissolved protein after the separation of supernatant.

II.2.2.3 Protein Solubilization by Liquid-Liquid Phase Transfer Method

The solubilization of proteins by liquid-liquid phase transfer method was performed by mixing the aqueous phase containing known concentration of protein with the carefully measured amount of reverse micellar solution in a tightly stoppered glass vial of 10 ml capacity. In each set of experiment, 2 ml of 1 mg/ml protein containing aqueous phase was mixed with 2 ml of 50 mM AOT/isooctane micellar solution for 20 minutes for the transfer of protein from the aqueous to the organic phase, and around 30 minutes if the back transfer from the organic to the aqueous phase was carried out. The resulting mixture was centrifuged at 2000 r.p.m. in temperature controlled Beckman high speed centrifuge for 20 minutes, to obtain a distinct phase boundary. Both the phases were separated carefully with the help of syringes and proteins were analyzed and estimated by standard methods. The percentage solubilization in

extracting phase was calculated w.r.t. the amount of protein present initially in the feed phase viz. 100% solubilization means 1 mg of protein was extracted by 1 ml of organic phase from 1 mg/ml of aqueous feed and vice versa.

II.2.2.4 Estimation of Proteins in Extracting Phase

The identification and determination of concentration of enzymes/proteins and other biomolecules in the extracting phase were carried out by the following experimental techniques.
(i) UV-vis Absorption method; (ii) Spectroscopic measurements; (iii) Lowry method and (iv) Electrophoresis.

UV-Vis Absorption Method

The protein concentration in both the phases were determined by measuring the uv absorption at 280 nm on Gilford-260 UV-Vis spectrophotometer at 30 $\pm$ 0.2^{o}C. The percentage solubilization of protein was calculated from the ratio of A_{280} values in the reverse micellar phase to the A_{280} of the feed aqueous phase, both values corrected for their base line readings. In both the cases, extinction coefficients for the proteins are assumed to be same [12].

Lowry Method

Folin-Lowry method [20] was used for the determination of protein concentration in aqueous phase. The absorbance of protein conjugated dye was measured at 560 nm on Gilford-260

.v.-vis spectrophotometer. BSA was used as the protein tandard. Determination of the protein in aqueous phases also ave the residual concentration of protein in the organic phase.

pectroscopic Measurements

The absorption spectra were recorded with a Gilford response '.V.-Vis spectrophotometer. Spectroscopic studies of the proteins ınd other biomolecules in the reverse micellar solution were :arried out against a reference cell containing the similar ıqueous solutions in the waterpool of the micellar system without :he protein/biomolecule. The characteristic spectra of the enzymes/proteins or biomolecules extracted from the mixture in desired aqueous or organic phase were compared to the authentic samples in the same phase. The spectroscopic method was used for the identification of particular component isolated from the complex mixture, in the aqueous/organic phase.

Electrophoresis

In few cases SDS polyacrylamide gel electrophoresis was used for the identification of proteins separations from mixture according to the method reported by Laemmli [21].

II.3 Results and Discussion

In the present work, the following two methods have been employed to solubilize the proteins in reverse micellar solution, (i) Solid extraction method and (ii) Liquid-liquid phase transfer

ethod. The third method, i.e. injection method is normally mployed for the solubilization of high molecular weight iopolymers, as the earlier two methods are suitable for the olubilization of low molecular weight biomolecules, and usually re not suitable for high molecular weight nucleic acids, in articular DNA [11].

I.3.1 Solid Extraction Procedure

The dry powder of enzymes/proteins was kept in contact with reverse micellar solution at particular W_o and pH of the aqueous solution transferred into micellar system. The percent solubilization of protein in micellar organic media was calculated with respect to the known concentration of same protein in aqueous solution. The absorption spectra of protein containing reverse micelles was recorded with reference to reverse micelles without protein at experimental W_o and pH. This method is quite useful for the solubilization of lipophilic protein such as Folch-Pi-Protein [22].

The solid extraction behavior of three proteins namely Pepsin (M_r 35000), Bovine Serum Albumin (M_r 65000) and Lipase (M_r 100000) using cationic reverse micelles of CTAB in $CHCl_3$-isooctane (1:1, v/v) and anionic reverse micelles of AOT in isooctane were studied. In each case, both the protein concentration and the water content at equilibrium in the supernatant organic phase were monitored carefully. The effects of W_o([H_2O]/[Surfactant]), pH and surfactant concentrations on

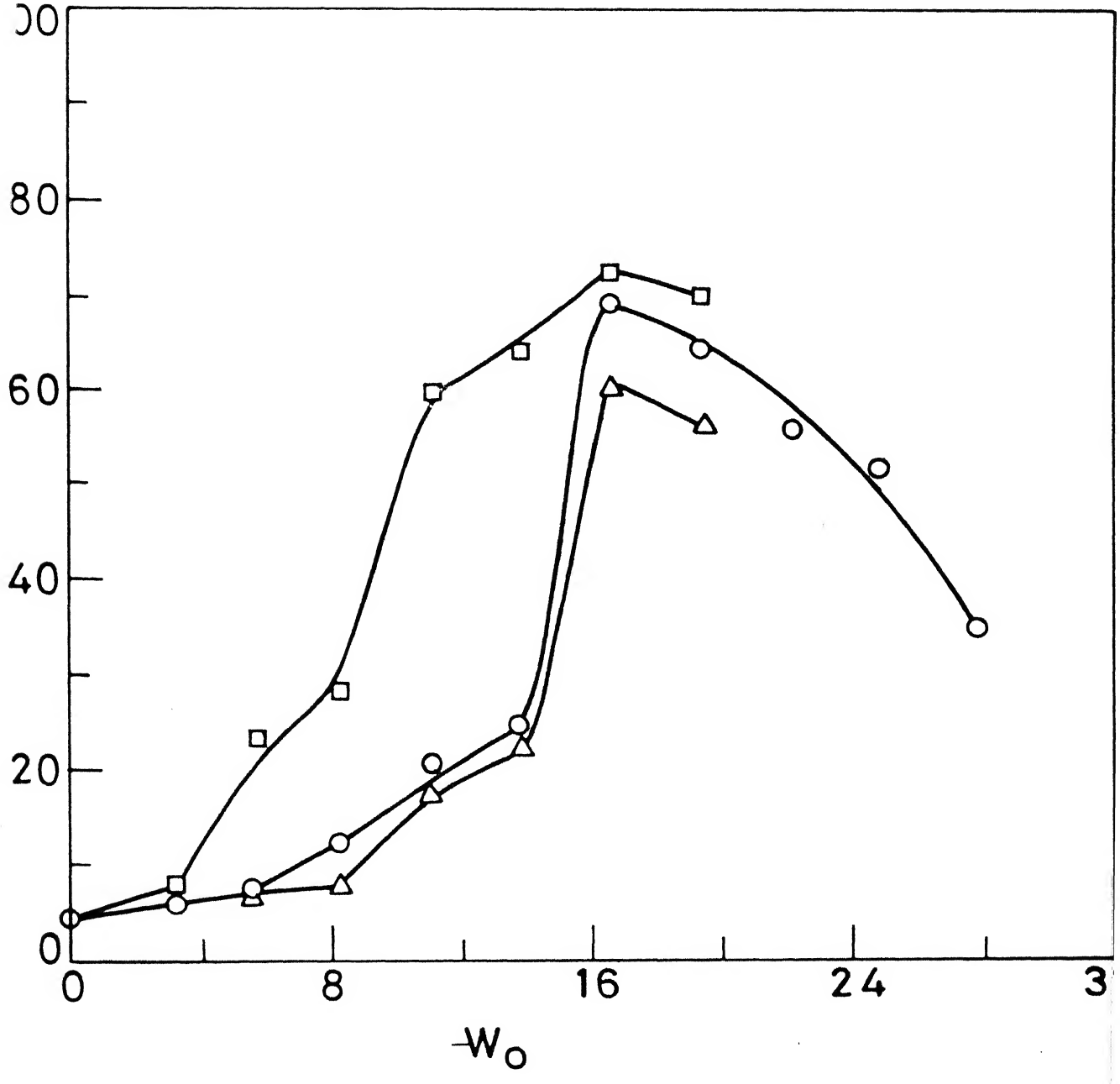


Fig. II.1. Variation of the concentration of solubilized BSA in 0.1 M CTAB/$CHCl_3$-isooctane (1:1, v/v) as a function of W_o at different pH conditions: (-O-O-) water, (-△—△-) pH 5.0 (citrate buffer), (-□—□-) pH 9.0 (glycine-KOH).

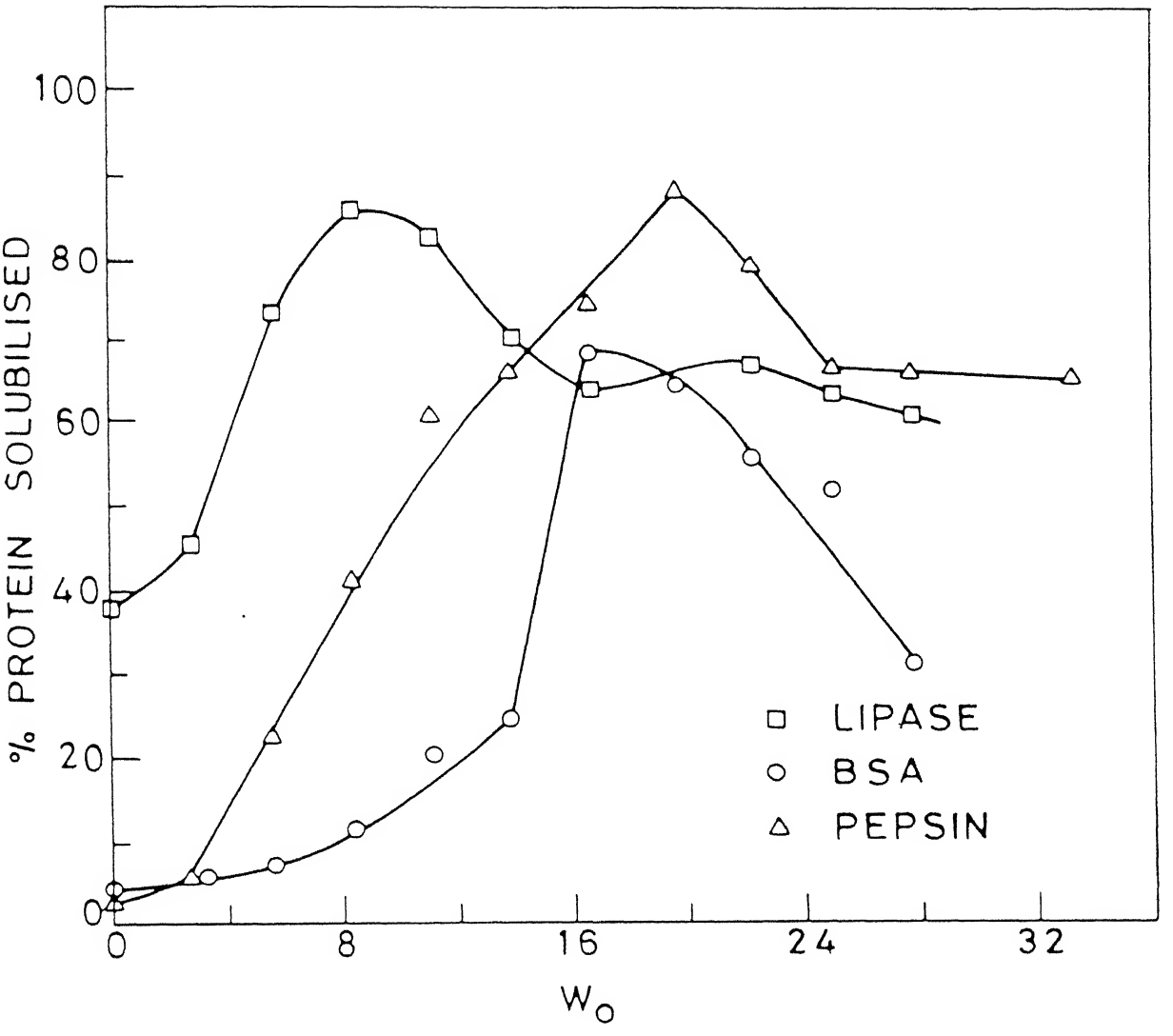


Fig. II.2. Variation of percent solubilization of proteins into 0.1 M CTAB/$CHCl_3$-isooctane (1:1, v/v) as a function of W_o at pH 9.0 (25 mM glycine-KOH): (-△—△-) pepsin; (-O-O-) BSA; (-□—□-) lipase.

the solubilization of the proteins in the micellar system were remarkable. The extraction behavior of the three proteins differed considerably from one another.

II.3.1.1 W_o Effect

The solubilization of protein is affected by the change in water content in the reverse micelles. Fig. II.1 shows the solubilization of BSA (expressed in term of concentration of protein) as a function of W_o at different pH in 0.1M CTAB/$CHCl_3$-isooctane (1:1, v/v). The protein solubilization increases with increase in W_o, attains a maximum and after W_o ~ 16 it begins to decrease. The solubilization plots under different conditions i.e. at pH 5.0 (citrate buffer) and pH 9.0 (glycine KOH) show that the solubilization is maximum at pH 9.0 which is above the isoelectric point (pI = 4.9) of BSA. At this pH, the protein is assumed to be negatively charged and maximum solubilization is favored due to the electrostatic interaction between the negatively charged protein and positively charged head groups of CTAB. The optimum W_o is 16 for all the cases.

Fig. II.2 represents the effect of W_o on the percentage solubilization of three proteins, Pepsin, BSA and lipase in 0.1M CTAB/$CHCl_3$-isooctane (1:1, v/v) with preadded water. The solubilization profile for all the proteins are different. In all the three cases, the solubilization of protein is highly dependent on W_o. The optimum W_o for the maximum solubilization of pepsin, BSA and lipase have been found to be 20, 16 & 8

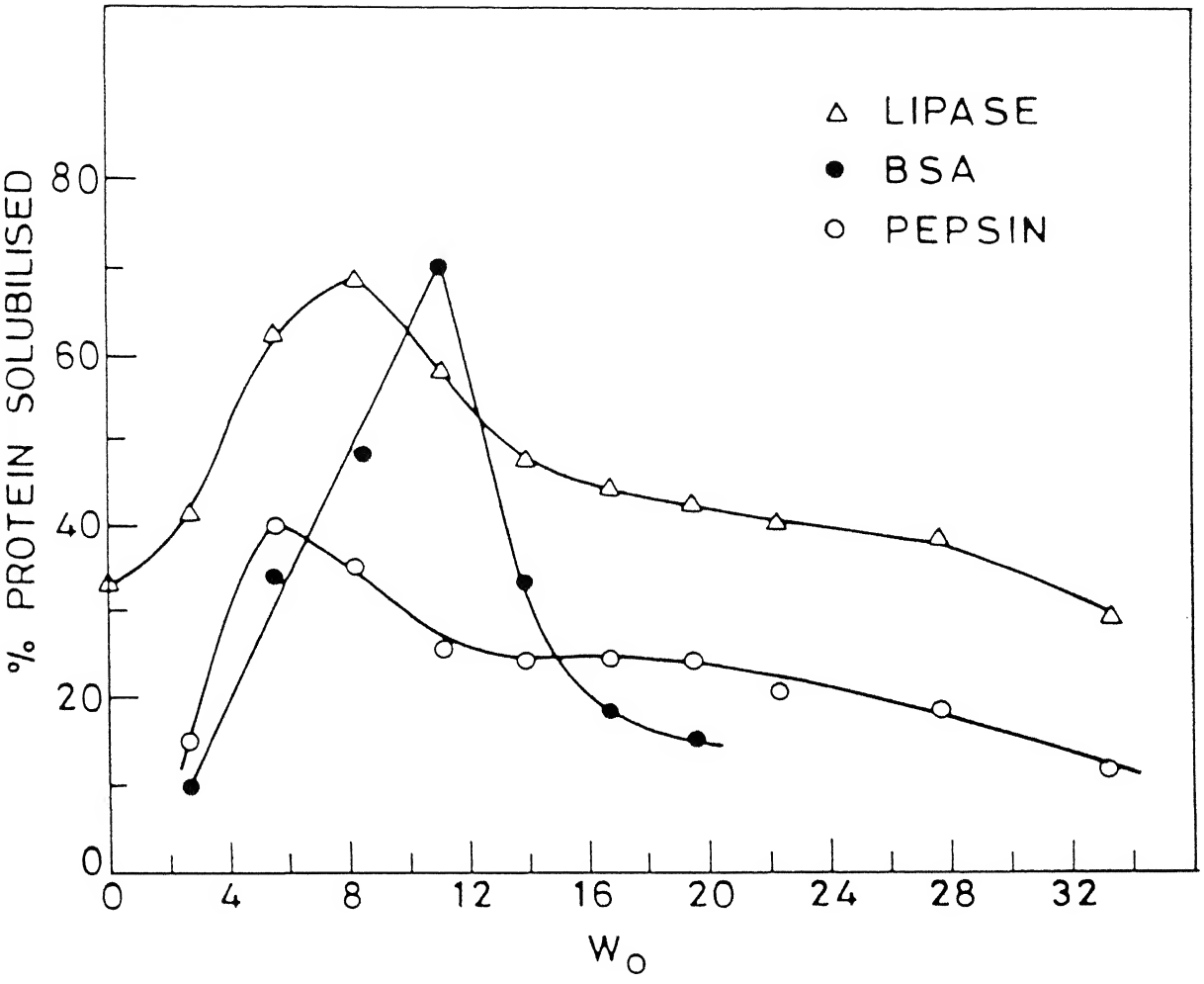


Fig. II.3. Variation of percent solubilization of proteins into 0.1 M AOT/isooctane as a function of W_o at fixed pH 6.4.

respectively. It appears interesting that in cationic reverse micellar system, the solubilization of high mol. wt. protein occurs at lowest W_o and vice versa.

In order to examine the solubilization behavior of these proteins in anionic reverse micelles the solubilization was studied as a function of W_o in 0.1M AOT/isooctane reverse micelles (Fig. II.3). The results show that the maximum solubilization of pepsin, BSA and lipase occurs at W_o value of 6, 11 and 8 respectively. Like the solubilization curve obtained in cationic reverse micelles, these proteins exhibit the similar trend of solubilization in AOT/isooctane. The remarkable point is that the solubilization occurs at low W_o range i.e. 6-11 rather than at high W_o values. However, the order of optimum W_o for highest solubilization of these proteins is different than that in cationic system. Here $W_{o,opt}$ of protein solubilization is not strictly in order of the mol. wt. of proteins. Pepsin, having least molecular weight (M_r 35,000) solubilizes at lowest W_o value ~6. In contrast high molecular wt. proteins lipase and BSA show maximum solubilization at W_o = 8 and 11 respectively. It should be noted that lipase from Candida cylindriccae is a dimer of 50,000 dalton. Maximum solubilization of lipase in AOT/isooctane at W_o 8 might be due to dissociation of this enzyme in organic media. Thus in 0.1M AOT/isooctane solution, the correlation for maximum protein solubilization appears to be the increase in W_o optimum values with the increase in molecular wt. of protein. In these experiments the stress is not on the

limit of maximum solubilization of proteins, rather the data give an idea about the solubilization characteristics of these proteins in both cationic and anionic reverse micelles.

Comparison of the results of Fig. II.2 and Fig. II.3, brings out the following interesting phenomena. The solubilization characteristics vary for different proteins. The case of lipase seems to be quite different as maximum solubilization of this protein occurs at W_o=8 in both the cationic and anionic reverse micelles. The solubilization of proteins is favoured at low water content inside the micelles. This result is in agreement with the recently reported data of Luisi et al. [23] for lysozyme where the maximum solubilization occurred at W_o ~8. The surprising finding in all the cases is the occurrence of maximum solubilization under conditions where there is relatively little free water in the waterpool of the reverse micelles. As discussed earlier (chapter I), water under these conditions is supposed to be bound to the polar internal walls of the micelles. In other words there should be no "free water" at W_o values below ~ 7 to 8. However, the fact remains, that the maximal extraction of the protein takes place at a tiny concentration of free water in reverse micelle. In turn, this indicates that novel solubilization capacity is one of the novel physical properties possessed by the water of the waterpool in comparison to bulk water. The reason behind this unusual phenomena is not yet clear. Further investigation on diverse proteins will probably give a sound basis for understanding the mechanism of solubilization.

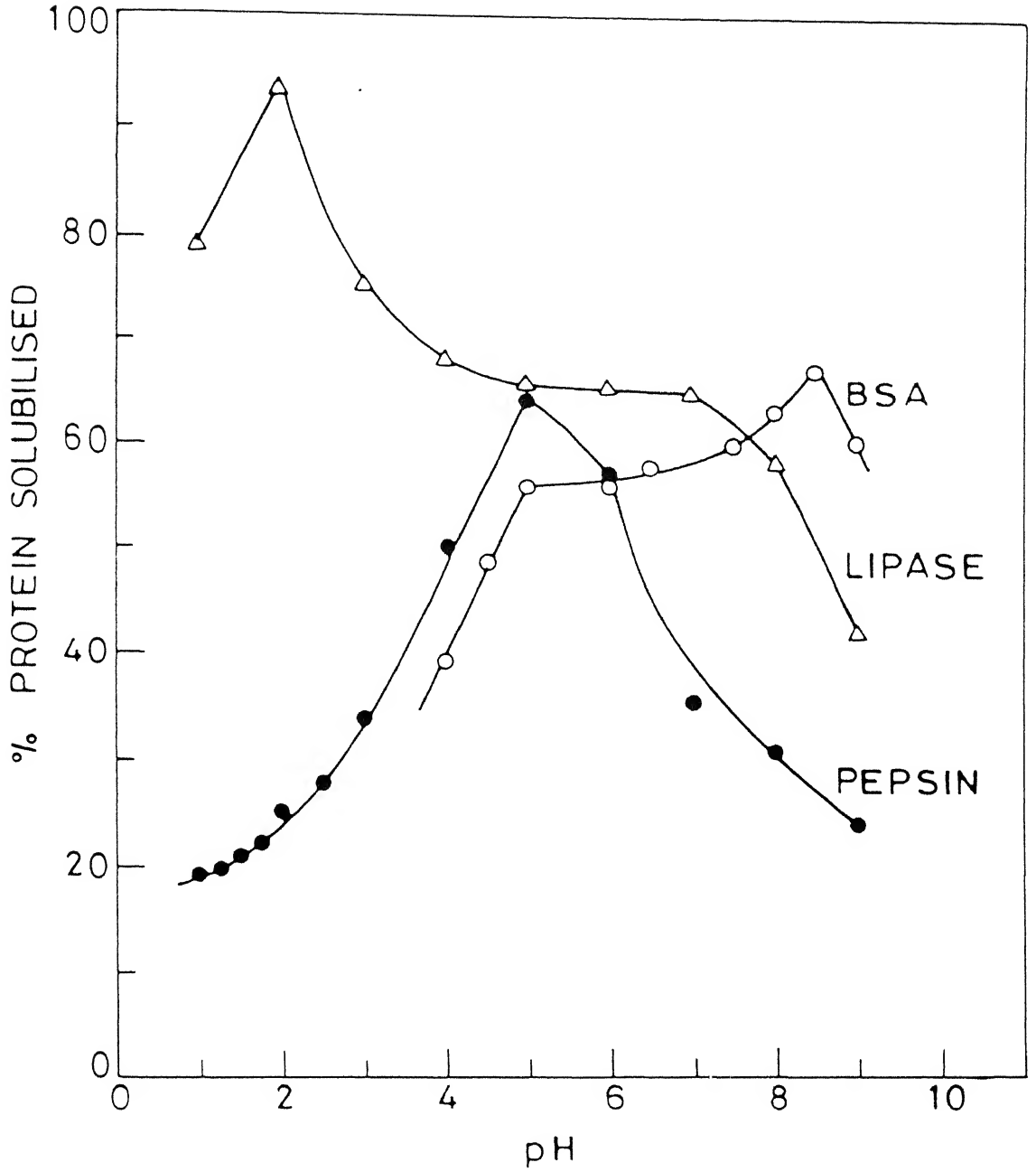


Fig. II.4. Variation of percent solubilization of proteins as a function of pH at fixed $W_{o,opt}$ in 0.1M CTAB/$CHCl_3$-isooctane for different proteins.

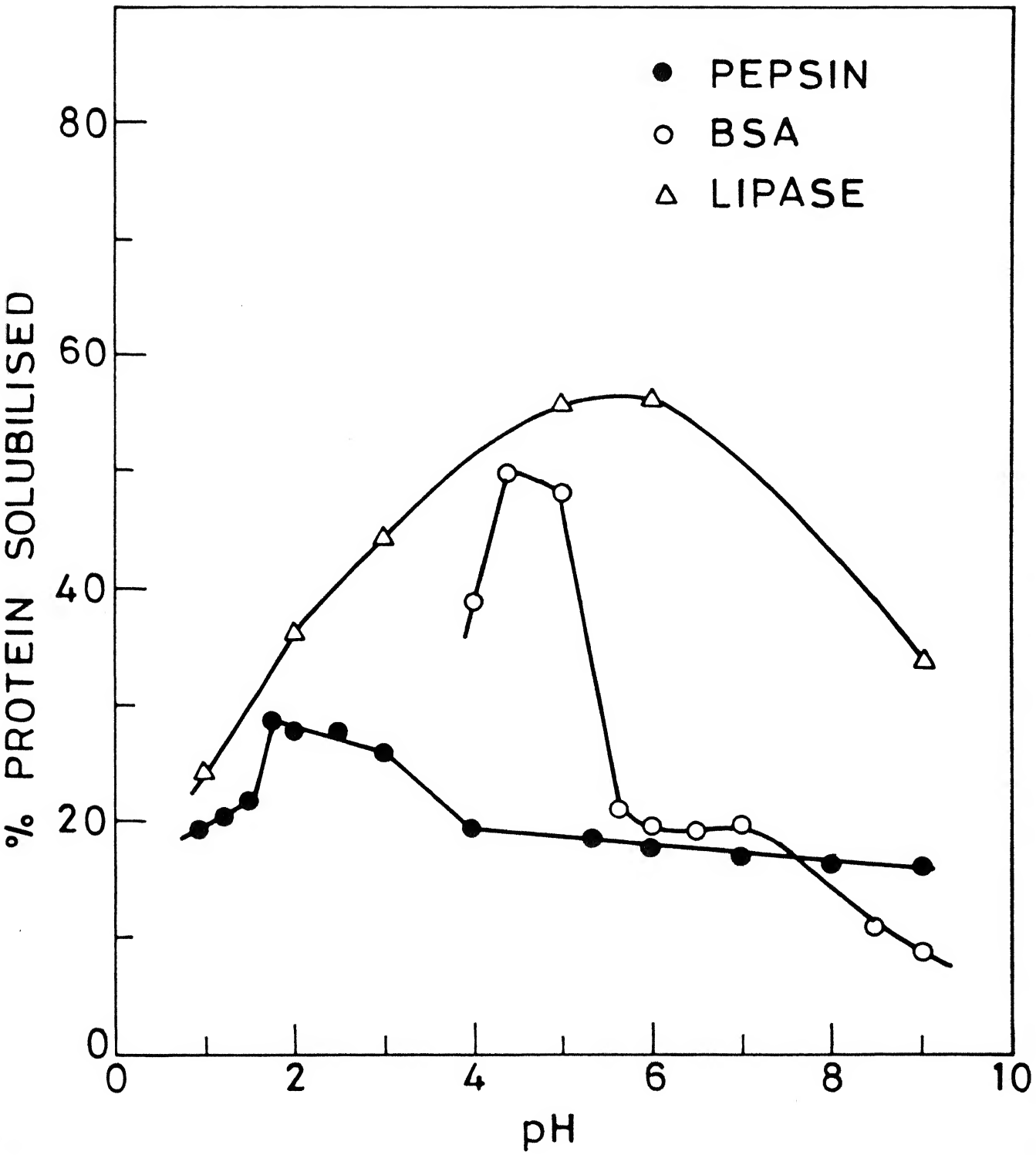


Fig. II.5. Variation of percent solubilization of proteins with the change in pH of preadded water into 0.1M AOT/ isooctane at the respective $W_{o,opt}$.

II.3.1.2 Effect of pH

The solubilization of protein is also dependent on pH. The pH dependence of the solubilization of these proteins into both cationic and anionic reverse micelles have been investigated. Here, the pH of the reverse micelles is referred to the pH of the water added inside the reverse micelles to maintain the particular W_o. Fig. II.4 shows the variation of percentage solubilization of proteins Pepsin, BSA & lipase as a function of pH of water added into 0.1M CTAB/$CHCl_3$-isooctane reverse micelles at their corresponding $W_{o,opt}$ values of 20, 16 & 8 respectively. The solubilization trend is different for all proteins. The optimum pH for the maximum solubilization of Pepsin (pI < 1.1), BSA (pI ~ 4.9) and lipase (pI ~ 4.2) are around pH 5.0, 8.0 and 2.0 respectively. It is interesting to note that the maximum solubilization of proteins in this cationic reverse micelles occurs at the pH higher than the isoelectric point of these proteins (except lipase).

Fig.II.5 represents the solubilization of these proteins with the change in pH of pre-added water inside the anionic reverse micelles of 0.1M AOT in isooctane. The maximum solubilization of protein Pepsin, BSA and lipase have been found at pH ~ 1.6, 4.2 and 6.0 respectively. In this study, lipase shows the deviation from the usual trend of maximum solubilization that occurs below the pI of the protein in the anionic reverse micelles. From the data of Fig. II.4 and Fig. II.5, it is clear that in general, the solubilization of protein is maximum near the isoelectric point

of protein. Certain deviation from the trend of solubilization behavior of protein may arise due to different factors such as purity of surfactant, solvents, source of proteins and denaturation at very low and very high pH values. Luisi and coworkers have also reported similar behavior in their study on lysozyme solubilization in AOT/isooctane [23]. In our study, different nature of solubilization curve as a function of W_o have been observed in the case of pepsin in AOT/isooctane. These findings indicate already a very important clue for the solubilization of the protein from the solid state. Under such conditions, the maximum extraction power of the reverse micelles is at around W_o value of 6 to 8 for AOT/isooctane and 8 to 20 for CTAB/$CHCl_3$-isooctane. Though Luisi et al. have reported the maximum solubilization at lower W_o values in the case of small proteins, but the present study on lipase and BSA has shown that even relatively bigger proteins also exhibit the maximal solubilization at lower W_o values in AOT/isooctane. This study reveals that a kind of unusual "micellar solubilization" is involved here, namely one in which the main player is not the bulk water of the waterpool but rather the internal surface of the micelle as well as the characteristics of the proteins play an important role in the solubilization.

II.3.1.3 The Influence of Surfactant Concentration

The effect of surfactant concentration on the solubilization at fixed W_o and pH of the water transferred inside the waterpool

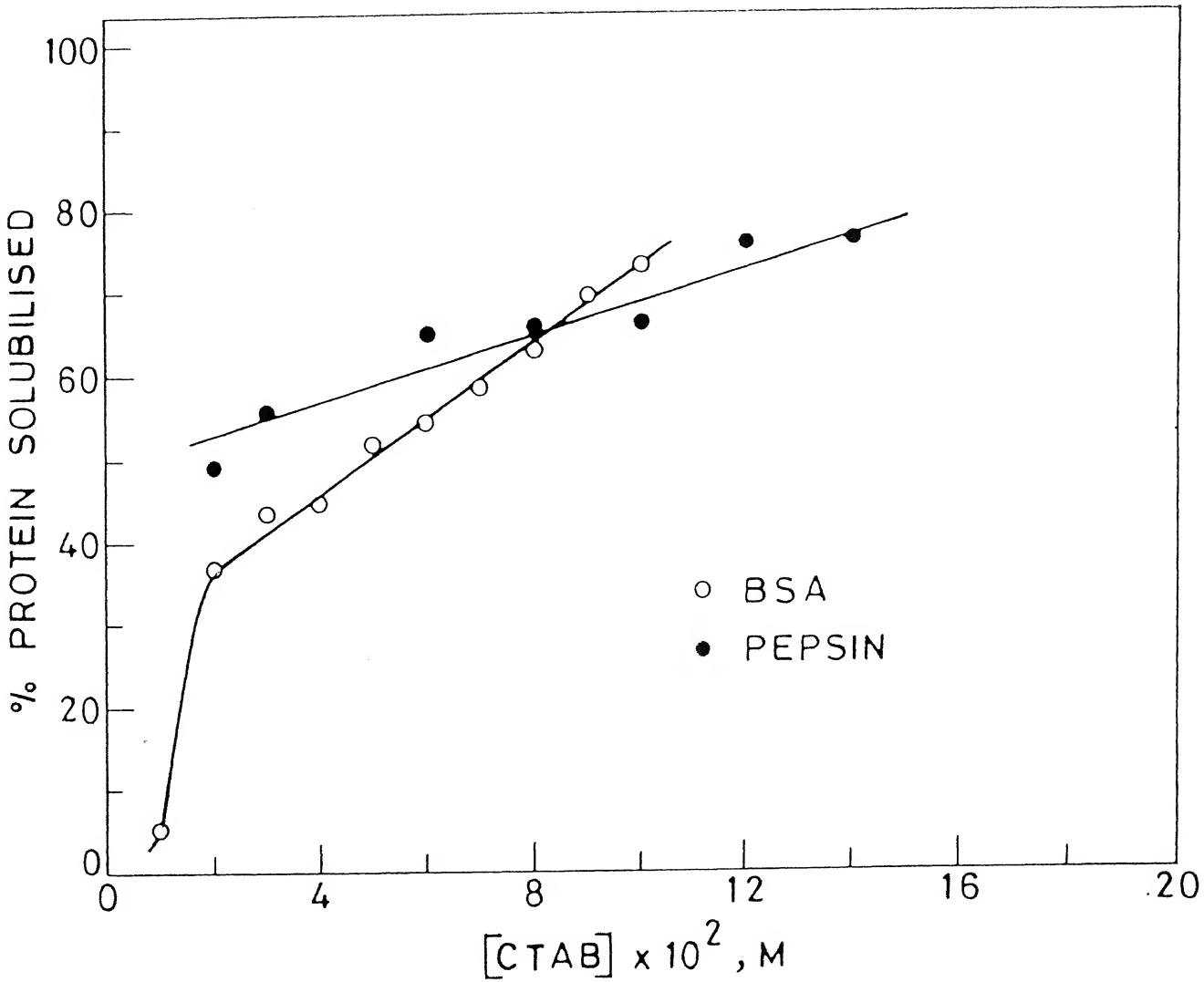
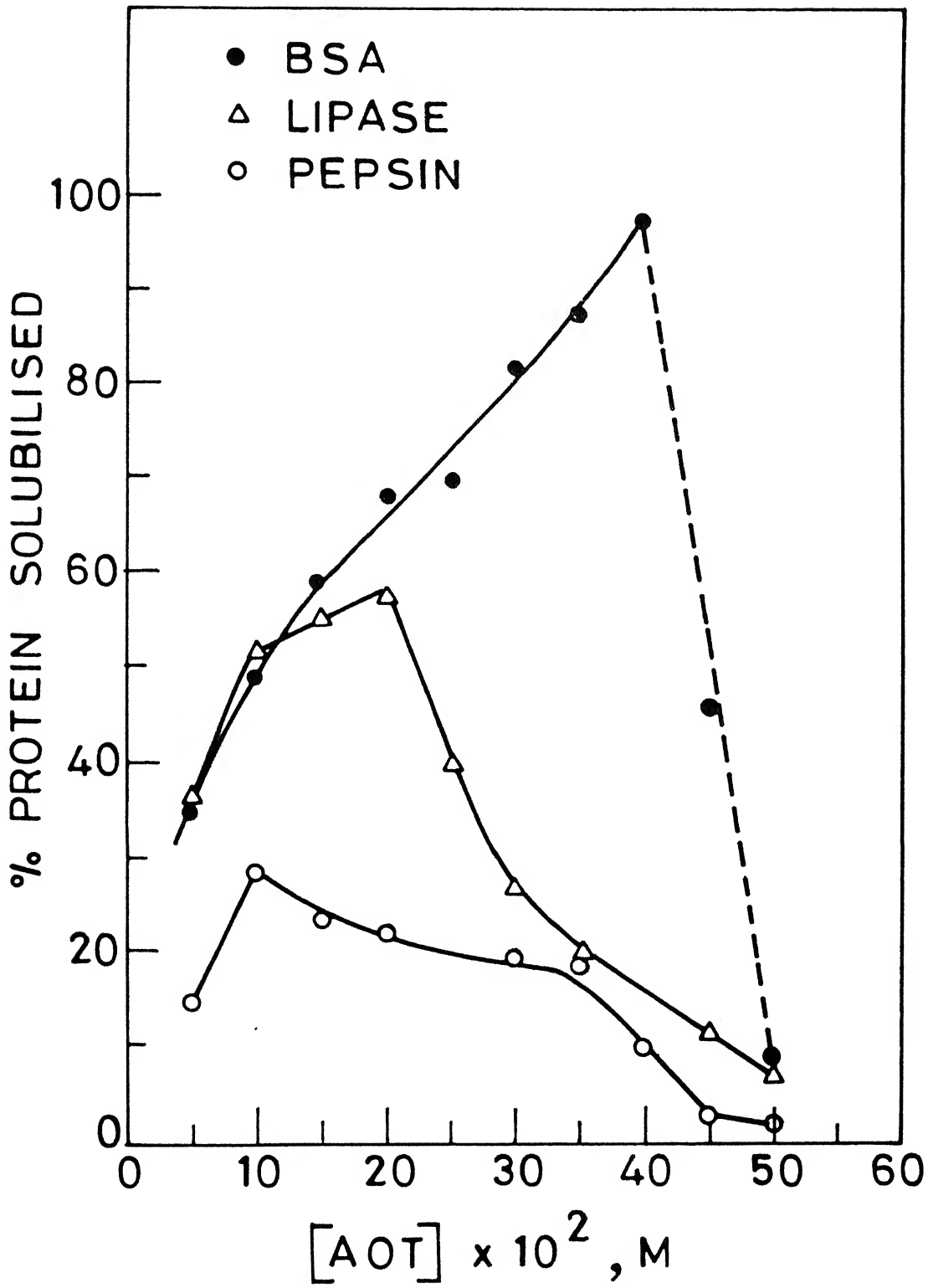


Fig. II.6. Dependence of percent solubilization of proteins into $CTAB/CHCl_3$-isooctane (1:1, v/v) reverse micellar solution as the function of CTAB concentration at fixed $W_{o,opt}$ and optimum pH.



g. II.7. Dependence of percent solubilization of proteins into the AOT/isooctane reverse micellar solution on AOT concentration at fixed optimum W_o and pH_{opt}.

of reverse micelles of these proteins was investigated. Fig. II.6 and Fig. II.7, show the dependence of solubility of proteins as a function of concentration of surfactant CTAB in $CHCl_3$-isooctane and of surfactant AOT in isooctane respectively at the corresponding optimum W_o and optimum pH for the single proteins. In each case, the solubilization of protein increases with the increase in surfactant concentration, and follows with decrease in solubilization after a certain concentration of the surfactant. The maximum solubilization of pepsin, BSA and lipase occur at 100 mM, 400 mM and 200 mM of AOT in isooctane respectively. The sharp decrease in solubilization of BSA after 400 mM concentration, appears to be due to the denaturation of protein. The increase of surfactant concentration leads to an increase in number of reverse micelles. Increased number of reverse micelles are able to solubilize increased amount of proteins. However on the other hand, the higher concentration of surfactant leads to denaturation of protein. The nature and magnitude of denaturation differs for different surfactants and proteins. At present it is difficult to assign the precise reason for denaturation of specific proteins, though it is realized that increased number of reverse micelles leads to increase in collisions between proteins and micelles which ultimately may induce unfavorable conformational changes or unfolding in proteins.

These studies indicate that the maximum extraction of protein by the reverse micelles from solid state, is remarkably

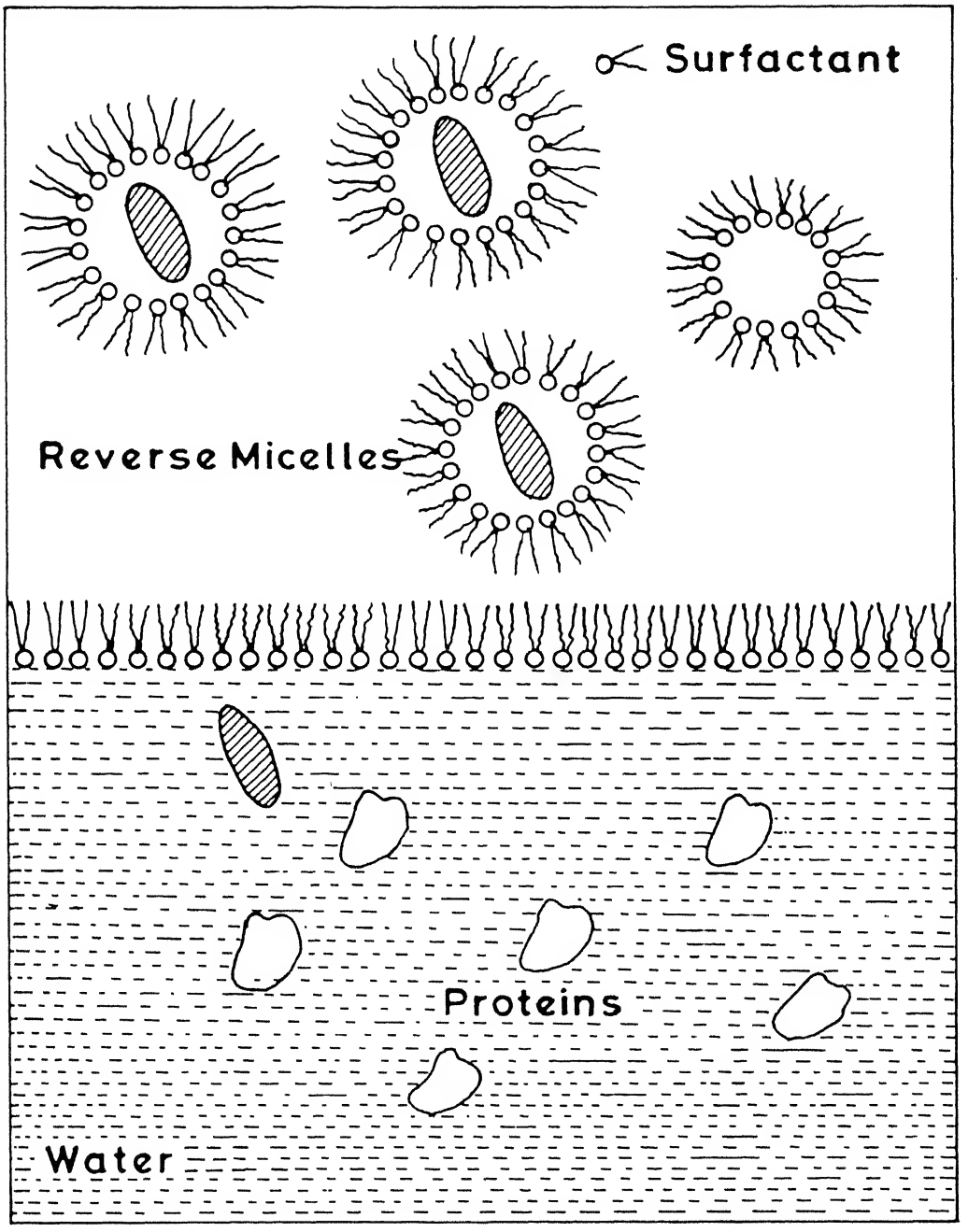


Fig. II.8. Schematic representation of the principle of selective solubilization of protein by reverse micellar phase from aqueous phase.

dependent on W_o, pH of waterpool (net charge of protein), surfactant concentration, nature and source of proteins.

II.3.2 Extraction of Proteins by Liquid-Liquid Phase Transfer Method

The liquid-liquid phase transfer is another method to solubilize proteins in apolar solvents via reverse micelles. In this method, the protein/biomolecule is initially contained in aqueous solution, is brought in contact with a supernatant of the surfactant in organic solution (e.g. AOT in isooctane). Depending upon the salt content in the aqueous phase and other parameters e.g. temperature, pH etc. the proteins are able to leave the aqueous phase and migrate into the micellar phase. In addition to these factors, solvent structure and the nature of surfactant, which play a significant role in solution, will influence the protein partitioning behavior in as much as they will affect the cooperative formation of the protein/micelle complex. Fig. II.8 systematically depicts the principle of selective solubilization by reverse micelles in organic phase from aqueous phase. Once, the particular enzyme/protein is completely solubilized in reverse micellar phase, as determined by monitoring the different parameters, it can be back transferred to the fresh aqueous phase.

The phase transfer process has been examined with various enzymes/proteins and biomolecules, such as cytochrome-C, pepsin, BSA, creatine kinase, lipase, alcohol dehydrogenase, cytochrome-C

eductase, coenzyme NADH and lipoic acid etc. In each case the rotein concentration was determined in the supernatant ydrocarbon solution at equilibrium. This study has been carried ut in the presence of KCl salt (at least 0.05M) in the aqueous hase. At lower salt concentration less than 0.05M, the micellar olution becomes cloudy, due to excessive water being transferred nto the reverse micelles. All experiments have been carried out ith stable, transparent micellar solution. The effects of pH nd salt concentration on the solubilization of various nzymes/proteins and biomolecules in 50 mM AOT/isooctane reverse icellar solution have been investigated to explore the potential for employing the phase transfer technique in the large scale recovery and concentration of proteins using liquid-liquid extraction.

II.3.2.1 Effect of pH on Protein Solubilization

The pH of the solution should affect the solubilization characteristics of a protein primarily in the way in which it modifies the charge distribution over the protein surface. Secondary factors, such as changes in protein conformation as a result of pH changes, may also play a role in this regard. At pH values below its isoelectric point (pI), or point of zero net charge, the protein will acquire a net positive charge, while above its pI, the protein will be negatively charged. Thus, if electrostatic interactions are the dominant factor in the solubilization process, solubilization should be possible with

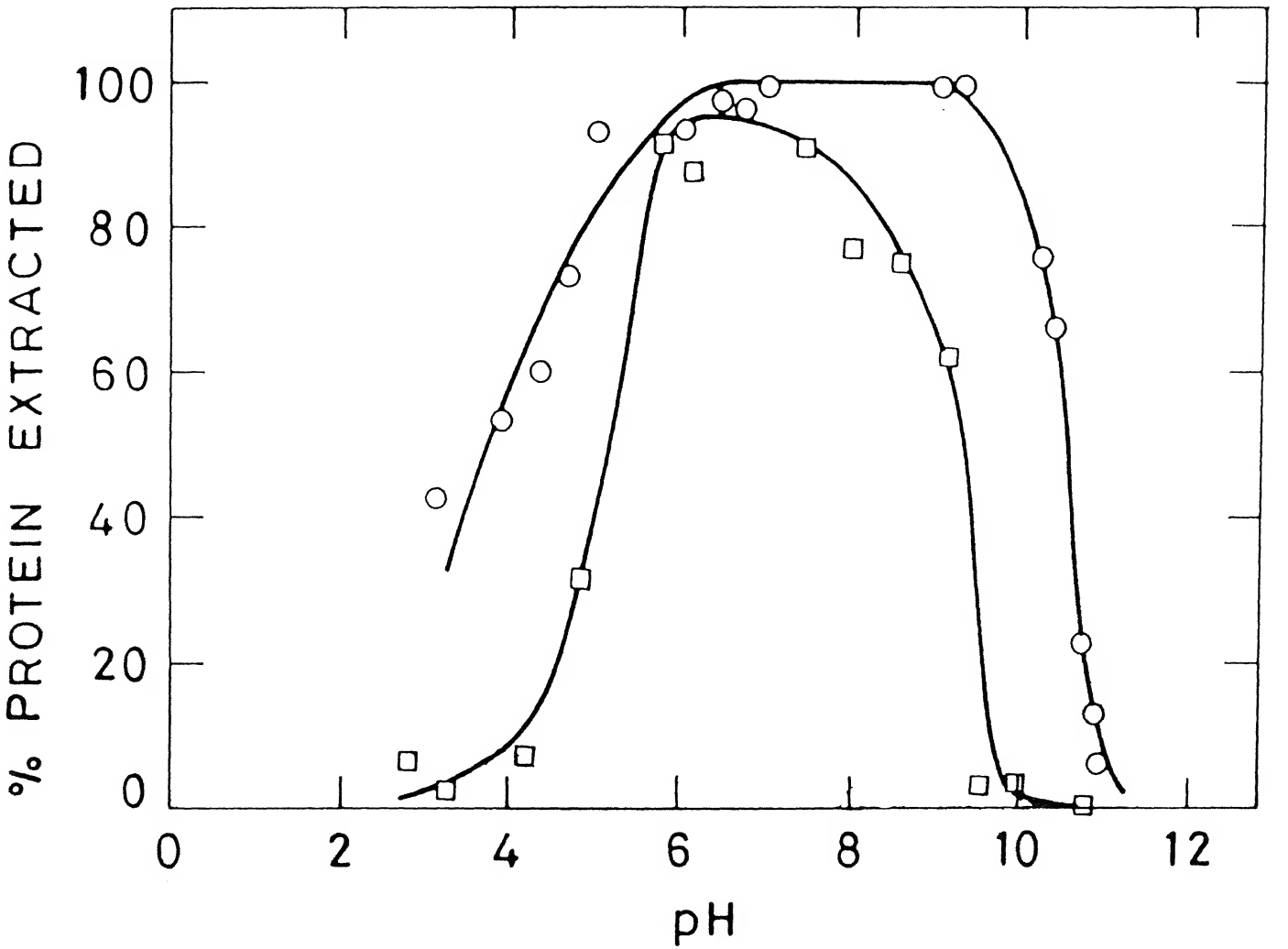


Fig. II.9. Effect of pH on the transfer of: (-o-o-) cytochrome-C, and (-□-□-) creatine kinase in AOT/isooctane reverse micellar solution from aqueous solution containing 0.1 M KCl.

TABLE II.1: Maximum Solubilization of Enzymes/Proteins and other Biomolecules into 50 mM AOT/Isooctane from 0.1 M KCl solution by liquid-liquid Phase Transfer Method.

Protein	Molecular weight	pI	% Solubilization	pH
Cytochrome-C	12,380	10.6	100	6.0-10.0
Elastase	25,900	8.5	~90	6.0- 7.0
Pepsin	35,000	<1.0	~30	2.5- 3.0
Peroxidase	40,000	7.2	~20	at all pH
Bovine Serum Albumin	65,000	4.9	Not at all	any pH
Creatine Kinase	81,000	6.1	97	5.8-7.0
Lipase from C. cylindraccae	1,00,000	4.2	47	2.75
Alcohol dehydrogenase (Yeast)	1,41,000	6.8	50	6.44
Cytochrome-C reductase	80,000		50	2.0
Biomolecules		**pK_a**		
Lipoic acid	206	4.7	95 99	3.0 (1.0 M KCl)
Nicotinamide Adenine dinneleotide (NADH)	783		Not at all	6.0-10.0

Experiment was done from aqueous feed as 0.1 M KCl of 1 mg/ml concentration. 100% solubilization means transfer of 1 mg material in 1 ml 50 mM AOT/isooctane. Experimental condition: temp.= ~30°C.

anionic surfactants only at pH less than pI of the protein, where electrostatic attractions between the protein and surfactant head groups are favorable. At pH above the pI, electrostatic repulsions would inhibit the protein solubilization. The reverse trends would be anticipated in the case of cationic surfactants. This becomes evident from the results of Hatton and coworkers [14,15] and the present study.

Solutions containing 1 mg/ml of protein and 0.1M KCl at various pH's were brought in touch with the micellar solution. Adjustment of pH was done by addition of 0.1M HCl or KOH solution to the protein solution. The pH values reported are those of protein containing aqueous feed before contact with the micellar phase. The pH values of the separated aqueous phase after contact were around 0.3-0.4 unit more than those of protein loaded aqueous phase before contact with micellar phase.

Fig. II.9 shows the percent of protein transferred from the feed solution to the micellar solution with varying pH, for cytochrome-C and creatine kinase. At high pH values, little or no protein is solubilized. As the pH of the system is lowered below the isoelectric pH of the proteins, which is given in Table II.1 there is a rapid change in solubilization, such that almost 100% of the protein is solubilized at lower pH values in the case of cytochrome-C and creatine kinase. For cytochrome-C, the maximum solubilization was maintained over a wide range of pH values from 6 to 10.5, although the maximum solubilization of creatine kinase is relatively narrow i.e. from 5.8 to 7.0. The

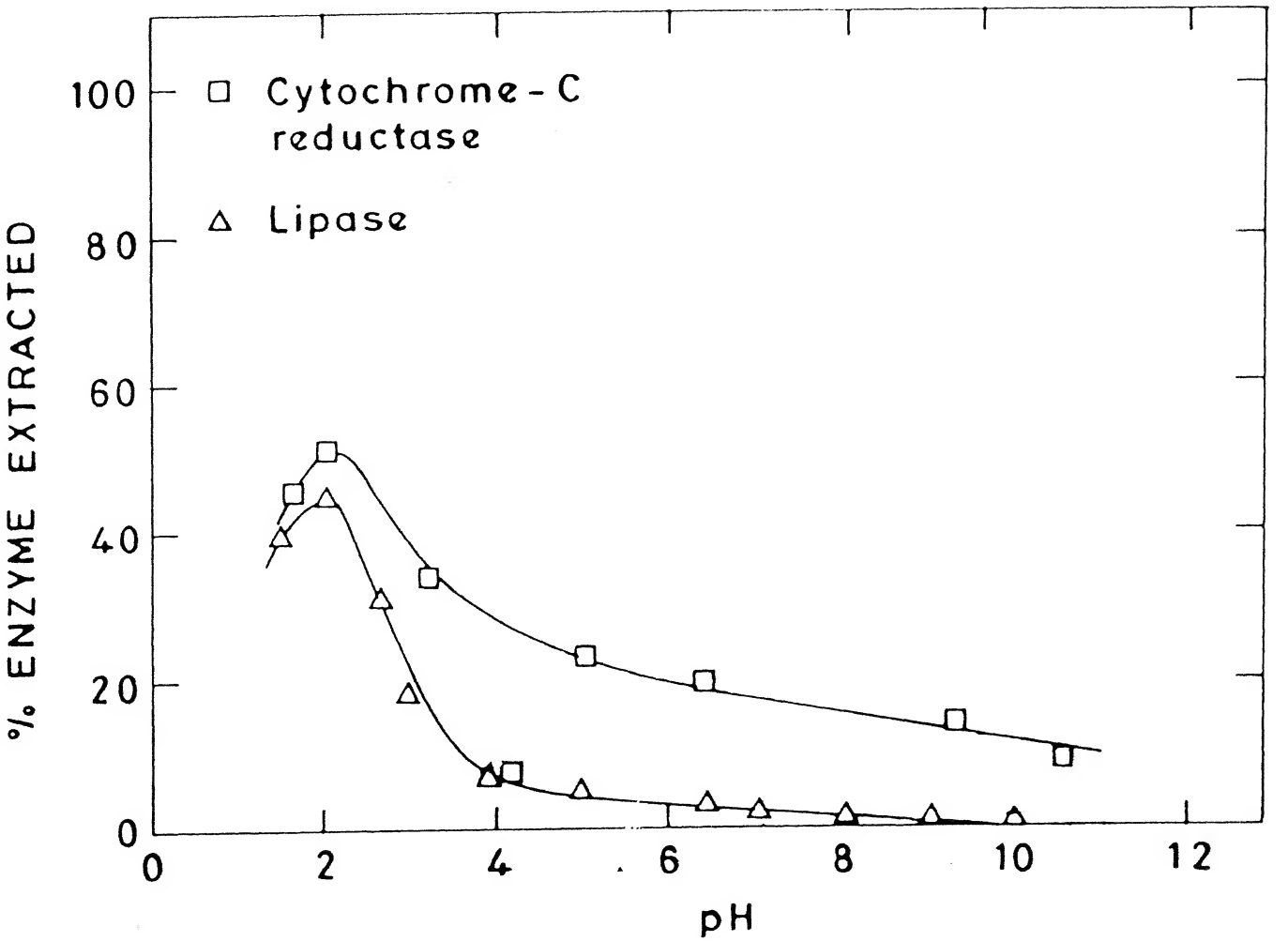


Fig. II.10. The effect of pH on the solubilization of:cytochrome-C reductase (-□-□-) and lipase (-Δ-Δ-) in 50 mM AOT/iso-octane reverse micellar solution. Salt concentration: 0.1 M KCl.

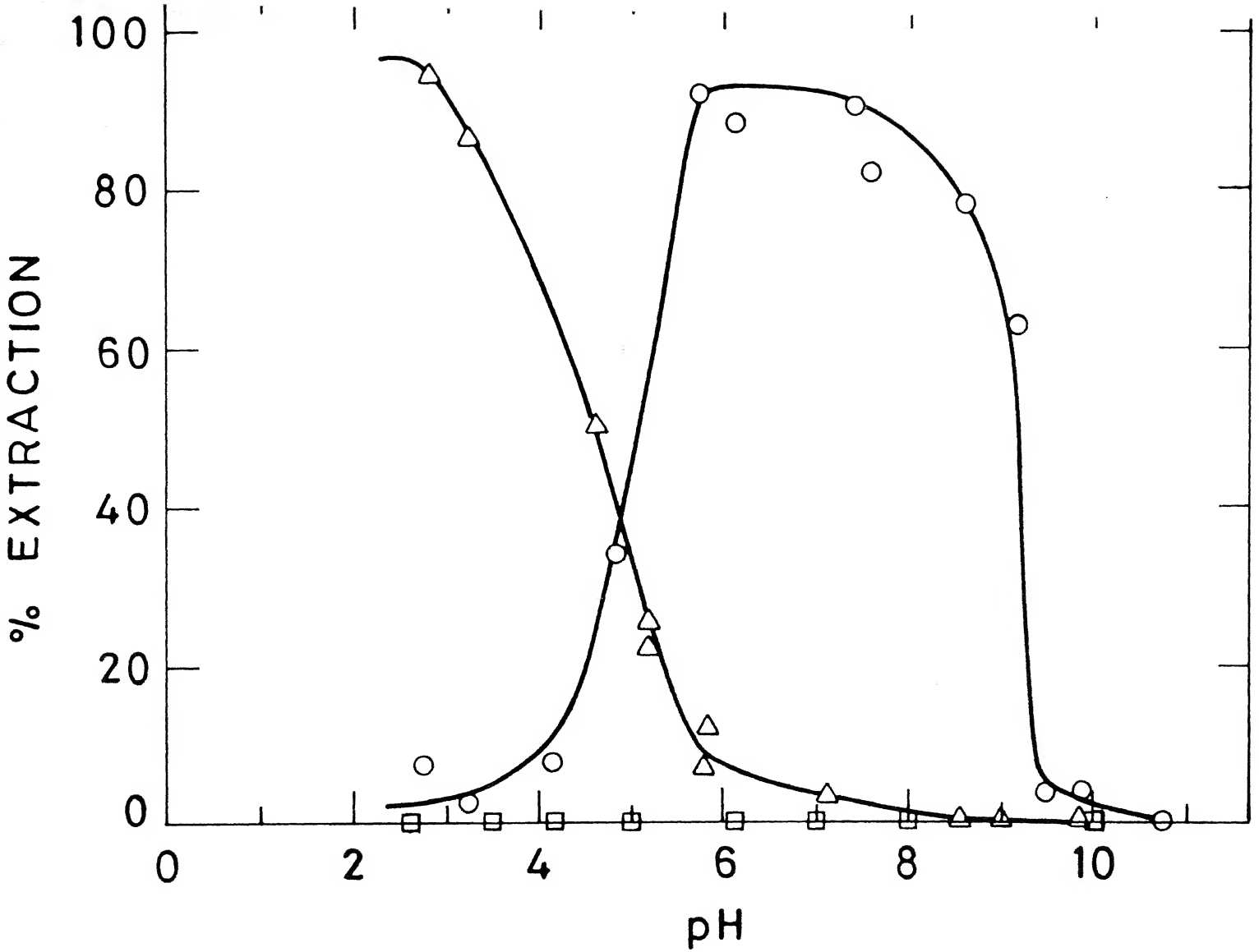


Fig. II.11. The effect of pH on the solubilization of: (-O-O-) creatine kinase, (-□-□-) bovine serum albumin and (-Δ-Δ-) lipoic acid in 50 mM AOT/isooctane reverse micellar solution. Salt concentration: 0.1 M KCl.

percentage solubilization is decreased again with lowering of pH below 5.0 in the case of cytochrome-C and creatine kinase.

Fig. II.10 represents the variation of percent of enzymes solubilized in 50 mM AOT/isooctane reverse micelles as a function of pH of enzymes dissolved in aqueous feed. The larger enzymes lipase, cytochrome-C reductase partially solubilize at low pH values below their pI values. The solubilization of lipase is completely diminished at the pH above than pI values (i.e. pH > 6.0). The enzymes cytochrome-C reductase and lipase are more susceptible to AOT surfactant. These enzymes are not completely solubilized at any pH value. Maximum solubilization of cytochrome-C reductase and lipase was ~50% at lower pH (around 2-3). A fraction of protein (~ 10-15%) is solubilized at any higher pH value (>9) in the case of cytochrome-C reductase. It appears that very large protein (i.e. high molecular weight) cannot be solubilized completely due to size exclusion effect.

The enzyme creatine kinase, a bigger & complex enzyme (mol. wt. 80,000, 2 sub units) completely solubilizes in the micellar solution at pH 5.8-7.0. The solubilization is sharply decreased at pH above 8.5. The decrease in solubility at very low pH values arises with the precipitation of protein at the junction of two liquid phases. But this solid mass again solubilizes at higher pH. Fig. II.11 shows many interesting features of these observations. Bovine serum albumin (M_r 65,000 dalton) does not solubilize in 50 mM AOT in isooctane at any pH value. Hatton and coworkers [13] have explained this by advocating in favour of size

exclusion effect due to the large size of BSA. It appears that the electrostatic interaction cannot be made sufficiently large by lowering the pH to counter act the other unfavorable factors which do not favor solubilization of protein BSA.

The solubilization of BSA in the reverse micellar solution of DDTAB (dodecyltrimethylammonium bromide) has also been carried out by us [24]. There is no solubilization at low pH (up to pH 8-9) but the solubilization of BSA sharply increases to about 60-70% at high pH in 0.1M DDTAB in $CHCl_3$-isooctane (1:1, v/v). These findings support the concept that in cationic reverse micelles, solubilization of protein takes place through a simple ion-pairing mechanism between the negatively charged BSA molecule and positively charged surfactant molecule.

The insolubility of BSA in 50 mM AOT/isooctane has been primarily attributed to the size exclusion factor. However, the case of complete solubilization of creatine kinase shows that for this protein electrostatic interaction overcomes the opposing effect of size exclusion factor. Lipoic acid [25] was also tested for the solubilization in micellar solution. The solubilization of lipoic acid in 50 mM AOT/isooctane is highly dependent on the pH of aqueous feed. Fig.II.11 shows its complete solubilization in 50 mM AOT/isooctane micellar solution at a low pH 3.0 in 0.1M KCl solution. With increasing pH the solubilization decreases and becomes zero above pH 9.0. Hatton et al. [26] have shown the combined effects of pH and ionic strength as protein solubilization. Their data demonstrated that the net

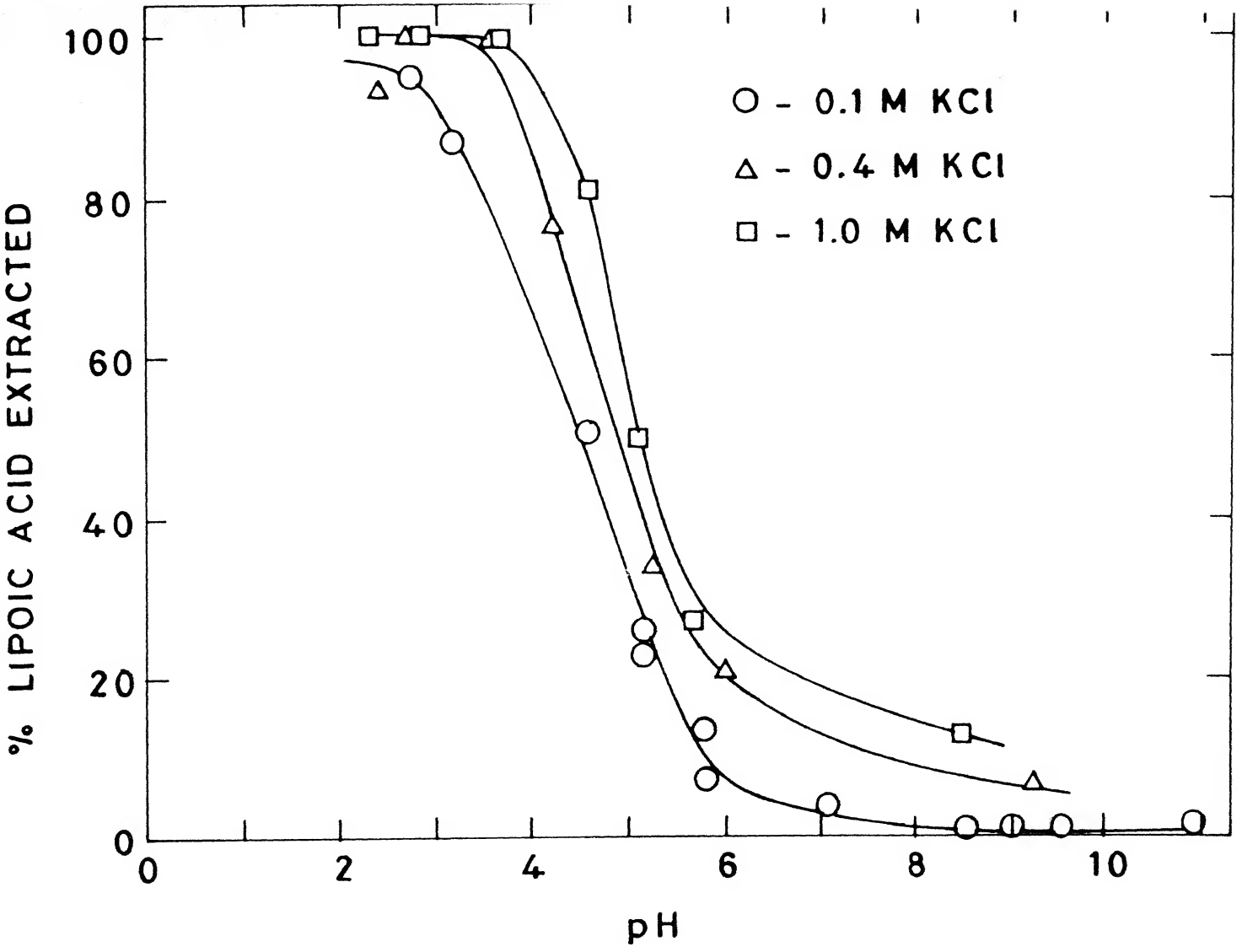


Fig. II.12. Effect of pH on the transfer of lipoic acid to 50 mM AOT/isooctane reverse micellar solution at different ionic strengths (KCl salt concentration).

effect of the increased salt concentration was to increase the pH excursion from the pI required for the occurrence of significant protein solubilization. The results for cytochrome-C were shown, in which the pH solubilization curve was found to be narrower at higher ionic strengths than at the lower salt concentrations. We were interested to find out the combined pH salt concentration effect on very small biomolecule, and compare the data with proteins obtained under similar conditions. Fig. II.12 depicts the change of solubilization of lipoic acid as a function of pH at varied KCl concentrations in aqueous feed. The [KCl] effect on the pH solubilization profile of lipoic acid was not significant. However, the range of maximum solubilization was slightly increased with the increase in KCl concentration. It seems that high ionic strength favors the electrostatic interaction between the lipoic acid and surfactant head, which leads to increase in solubilization. Table II.1 summarizes the maximum percent of solubilization of different biopolymers into 50 mM AOT/isooctane from the aqueous feed of single protein/ biomolecule containing 0.1M KCl solution. These studies show that in general, higher solubilization is achieved at pH values below the isoelectric point (pI) of proteins, probably due to strong electrostatic attraction between the positively charged proteins and the negatively charged AOT surfactant heads forming the inner micellar wall. Above the pI, the protein solubilization is diminished because of unfavorable electrostatic repulsions between the similar charges on protein and surfactant head. Despite the above data, phenomena of solubilization of very large

proteins, in 50 mM AOT/isooctane, cannot be rationalized in a straight forward way because of the reported observations from other studies that for larger proteins, size exclusion effect completely dominates over the electrostatic interaction.

II.3.2.2 Effect of Ionic Strength on Solubilization

The solubilization of proteins from one liquid phase to another one is affected in a number of ways by the ionic strength of the aqueous phase in contact with the reverse micellar solution. The first is through the mediation of the electrostatic interactions between the protein surface and the surfactant head groups through the characteristic change in electrical double layers adjacent to both the charged surface of protein and surfactant head. An increase in ionic strength compresses the range over which electrostatic interactions can overcome the thermal motion of the solute molecules, and thus decreases the protein/surfactant interactions, inhibiting the solubilization of the protein. The major effect of change in the ionic strength of the aqueous solution is expected to be the electrostatic screening of interactions between the charged groups on the protein surface and the surfactant head groups. These are reduced as the ionic strength is increased. Such variations may affect protein solubilization through the protein hydrophobic energy, and by shifting ionization equilibria.

A third effect of ionic strength is to salt-out the protein from the micellar phase because of the increased propensity of

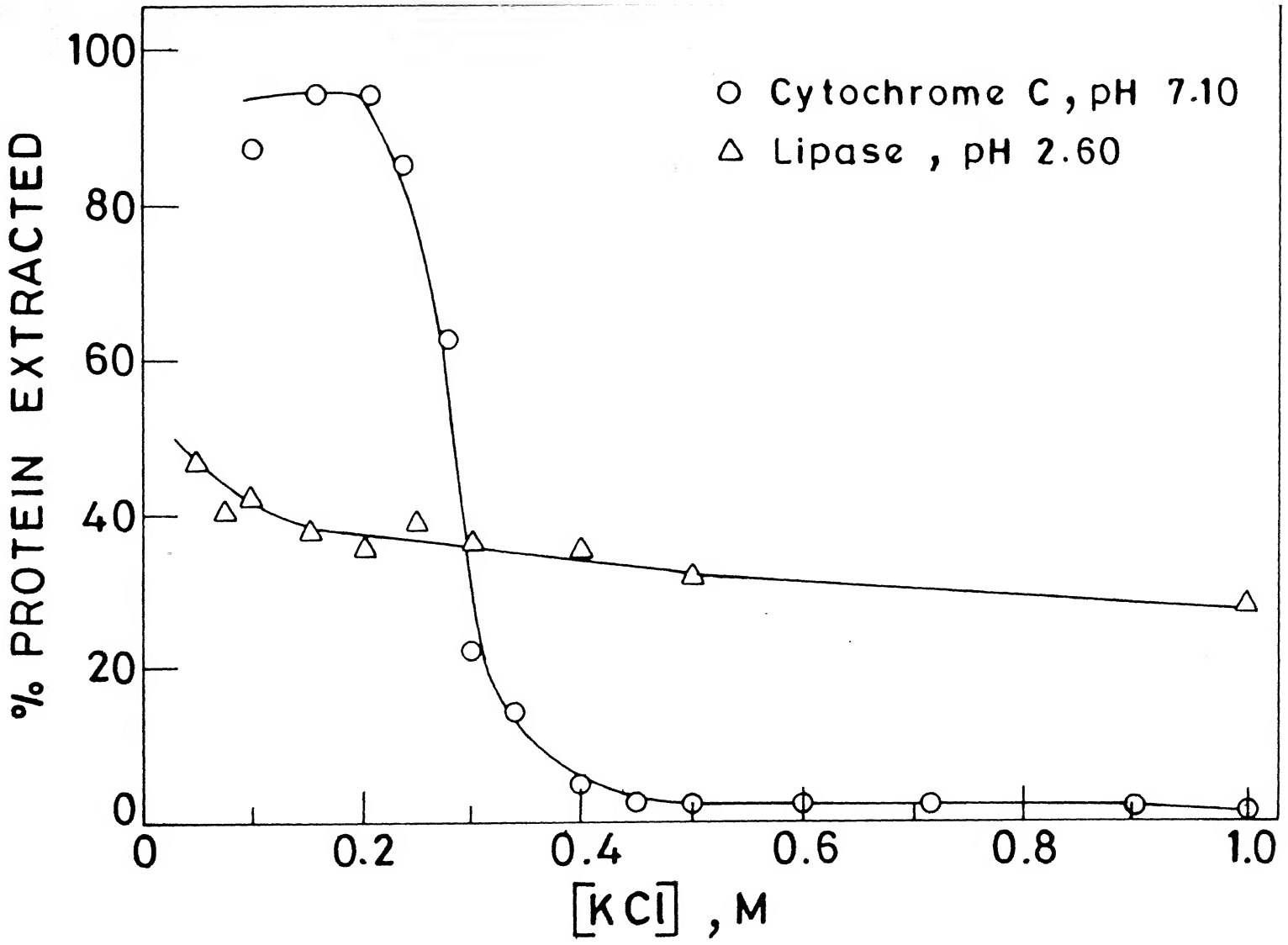


Fig. II.13. The effect of [KCl] on the solubilization of: cytochrome-C (-O-O-) and lipase (-Δ-Δ-) in 50 mM AOT/isooctane solution.

the ionic species to migrate to the micellar waterpools and to displace the protein.

Solutions containing 1 mg/ml of proteins (cytochrome-C, pH 7.1, lipase, pH 2.50) at varying concentrations of KCl were brought together with the reverse micellar solution, 50 mM AOT in isooctane. Fig. II.13 shows the percentage solubilization of proteins as a function of KCl concentration at fixed pH of the aqueous phase in contact with the micellar solution. Cytochrome-C was almost completely solubilized in the micellar solution at low ionic strength (0.1M) but there was no solubilization at high ionic strength (0.5M). The solubility profile shows the sharp transition, where the decrease in solubilization was predominant over a fairly narrow range of KCl (0.3M). The solubilization of lipase with the variation of [KCl] was not significantly affected. Lipase solubility in this AOT/isooctane solution from the aqueous phase containing 0.1M KCl was almost equal to that at 1.0M KCl solution. However, remarkable change in solubilization behavior of cytochrome-C has been observed with the change in [KCl]. The pH has profound effect on the solubilization of lipase at fixed concentration, 0.1M KCl (as shown in Fig. II.10). This observation shows that solubilization behavior of lipase in 50 mM AOT/isooctane is markedly affected by the electrostatic interaction due to pH change but not with change in [KCl]. It appears that this phenomenon is dependent on the nature of protein along with the protein net charge in the aqueous solution.

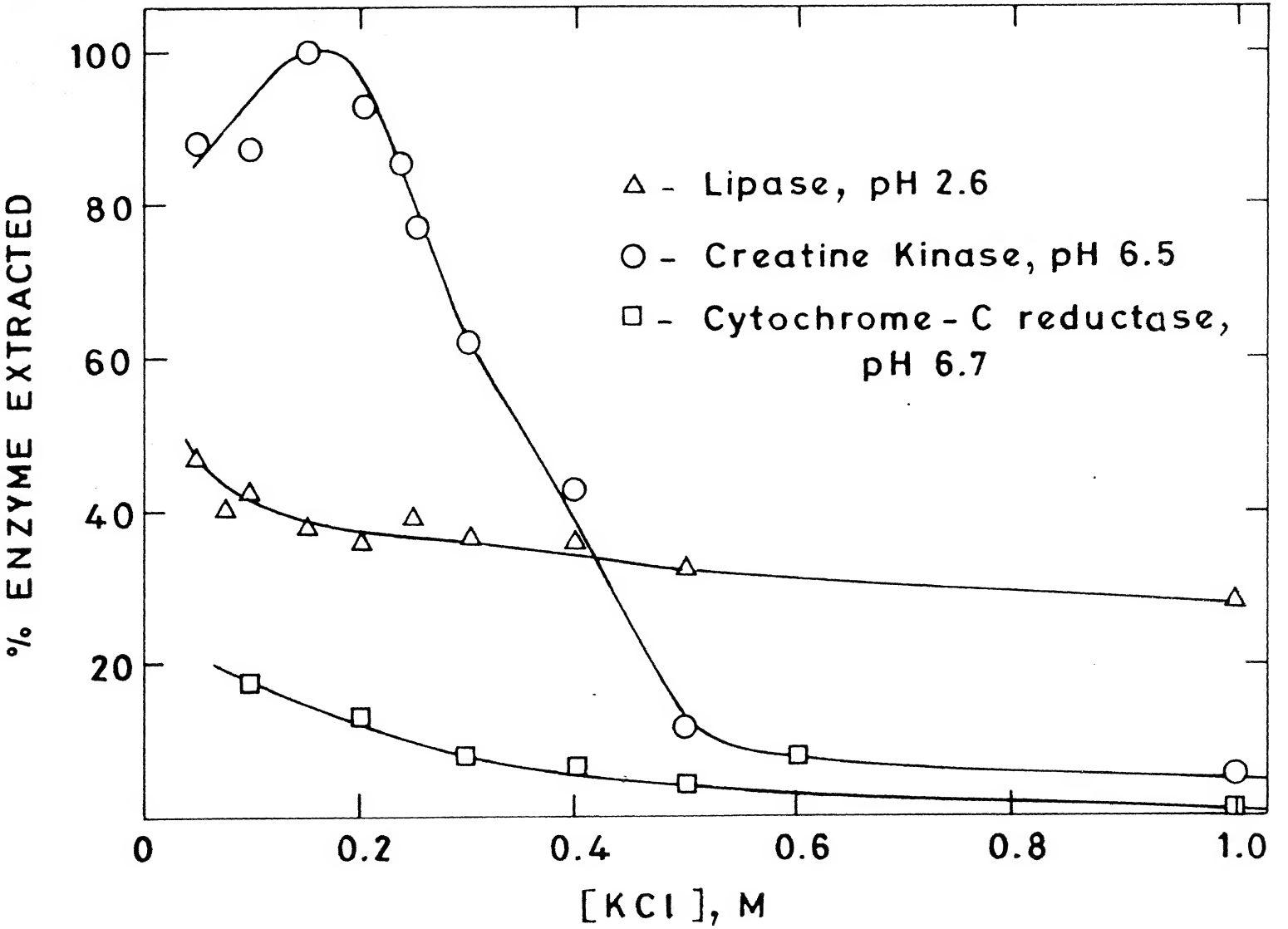


Fig. II.14. The effect of [KCl] on the transfer of lipase (-Δ-Δ-), creatine kinase (-O-O-), and cytochrome-C reductase (-□-□-) in 50 mM AOT/isooctane solution.

A more detailed study has been carried out with another two enzymes, cytochrome-C reductase and creatine kinase represented in Fig. II.14. This figure presents the effect of [KCl] in term of percent of solubilized enzymes in the micellar phase. cytochrome-C reductase does not show significant effect of KCl concentration on its solubilization and in this respect its behavior is similar to that of lipase. However, a sharp transition in the decrease of solubility of creatine kinase was observed between KCl concentration 0.2-0.5M. Thus the solubility of creatine kinase is highly dependent on the [KCl] in aqueous phase.

In general at low ionic strength and at pH below the pI of the protein, complete or maximum solubilization of proteins was observed. Increase in ionic strength appears to reduce the favorable electrostatic interaction between protein and micelle for proteins of all sizes at neutral pH. In addition ionic strength increase appears to interfere with the size exclusion effect expressed by large proteins. The salt concentrations at which abrupt/sharp decrease in extraction occurred are different for each protein.

II.3.3 Separation of Protein Mixtures

The effective utilization of the extracting phases for large scale product recovery depends on the partitioning behavior of the different components in the feed stream between the two phases and on process considerations such as the relative ease

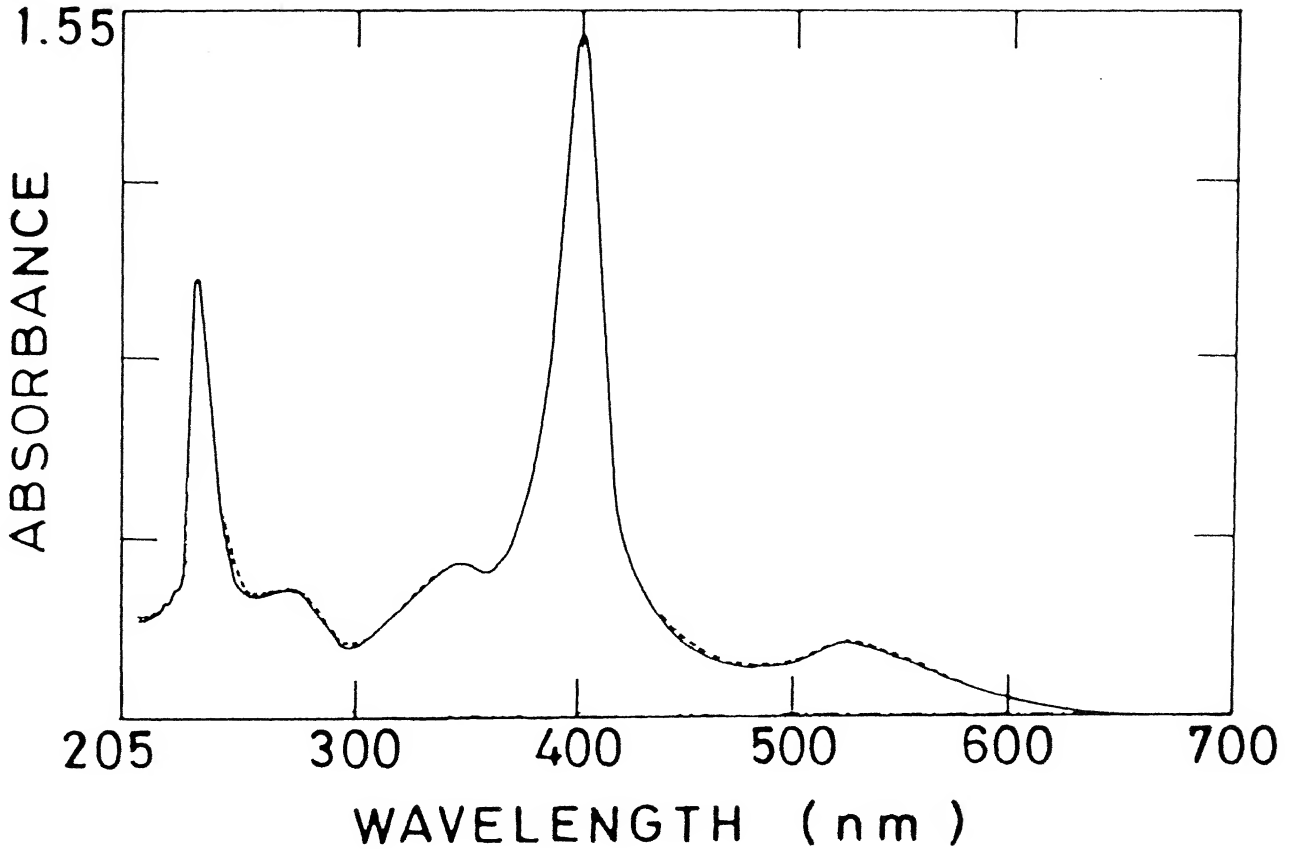


Fig. II.15(A). Absorption spectra of cytochrome-C in separation process: (———) in water, 0.1 M KCl, pH 10.0 (------) in organic phase, 50 mM AOT/isooctane after transfer of cytochrome -C from mixture (lipase & cytochrome-C containing) aqueous feed. Conditions are [cytochrome-C] = 0.5 mg/ml; [lipase] = 0.5 mg/ml.

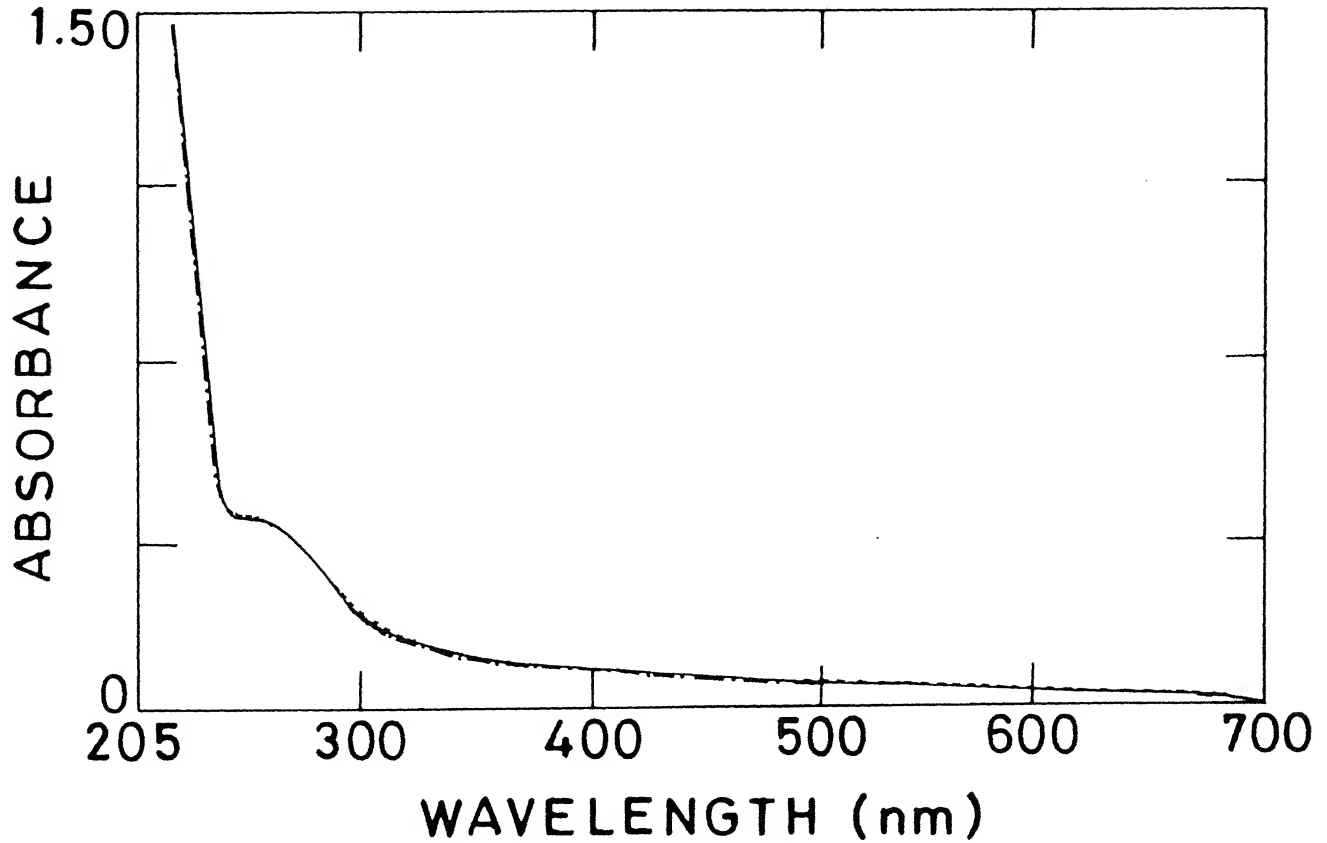


Fig. II.15(B). Absorption spectra of lipase: (——) in aqueous phase after the contact of lipase containing aqueous feed with 50 mM AOT/isooctane ; (·-·-·) retained in aqueous phase after the complete transfer of cytochrome -C in reverse micelles from binary mixture containing aqueous feed on contact with 50 mM AOT/isooctane. Conditions are same as in 15(A).

ABLE II.2: List of Various bio-mixtures that have been successfully resolved into single components by liquid-liquid extraction technique.

Binary Mixture

Cytochrome-C + Lipase; Creatine Kinase + Lipase; Cytochrome-C + NADH; Creatine Kinase + NADH; Cytochrome-C + Lipoic acid; Lipoic acid + BSA etc.

Ternary Mixture

Cytochrome-C + Creatine Kinase + Lipase

Creatine Kinase + BSA + Lipoic acid

Quart Ternary Mixture

Cytochrome-C + Creatine Kinase + BSA + Lipoic acid.

For binary mixture, the concentration of single component was 0.5 mg/ml. For ternary and quarternary mixture 0.33 mg/ml and 0.25 mg/ml were taken respectively.

with which the phases can be contacted and subsequently separated. The results presented earlier have demonstrated that solubilization characteristics of proteins are highly dependent on their charge -pH functionality and other factors which are not well understood. This knowledge of the solubilization behavior of single proteins has been used to separate a series of binary mixtures (like cytochrome-C + lipase, cytochrome-C + creatine kinase, creatine kinase + NADH etc.) and tertiary mixtures like creatine kinase + BSA + lipoic acid; cytochrome-C + creatine kinase + lipase. Table II.2 summarizes all the mixtures that have been separated using reverse micelles.

II.3.3.1 Binary Protein Mixture

An example of separation is shown in Figs. II.15(A) and II.15(B) which show the absorption spectra of authentic sample and the extracting phase containing cytochrome-C and lipase respectively. They provide the evidence for the separation of cytochrome-C from lipase as cytochrome-C shows two absorption maxima at 280 nm and 404 nm whereas lipase absorbs maximum only at 260 nm. In this case, the electrostatic interaction which helps in complete solubilization of cytochrome-C in 50 mM AOT/isooctane and prevents the solubilization of lipase at pH 7.0, 0.1M KCl of aqueous feed, is utilized for the separation. The cytochrome-C loaded organic phase contacted with 0.5M KCl, pH 10.0, yields back transfer of cytochrome-C in aqueous phase again. In the back transfer, the rate of extraction was

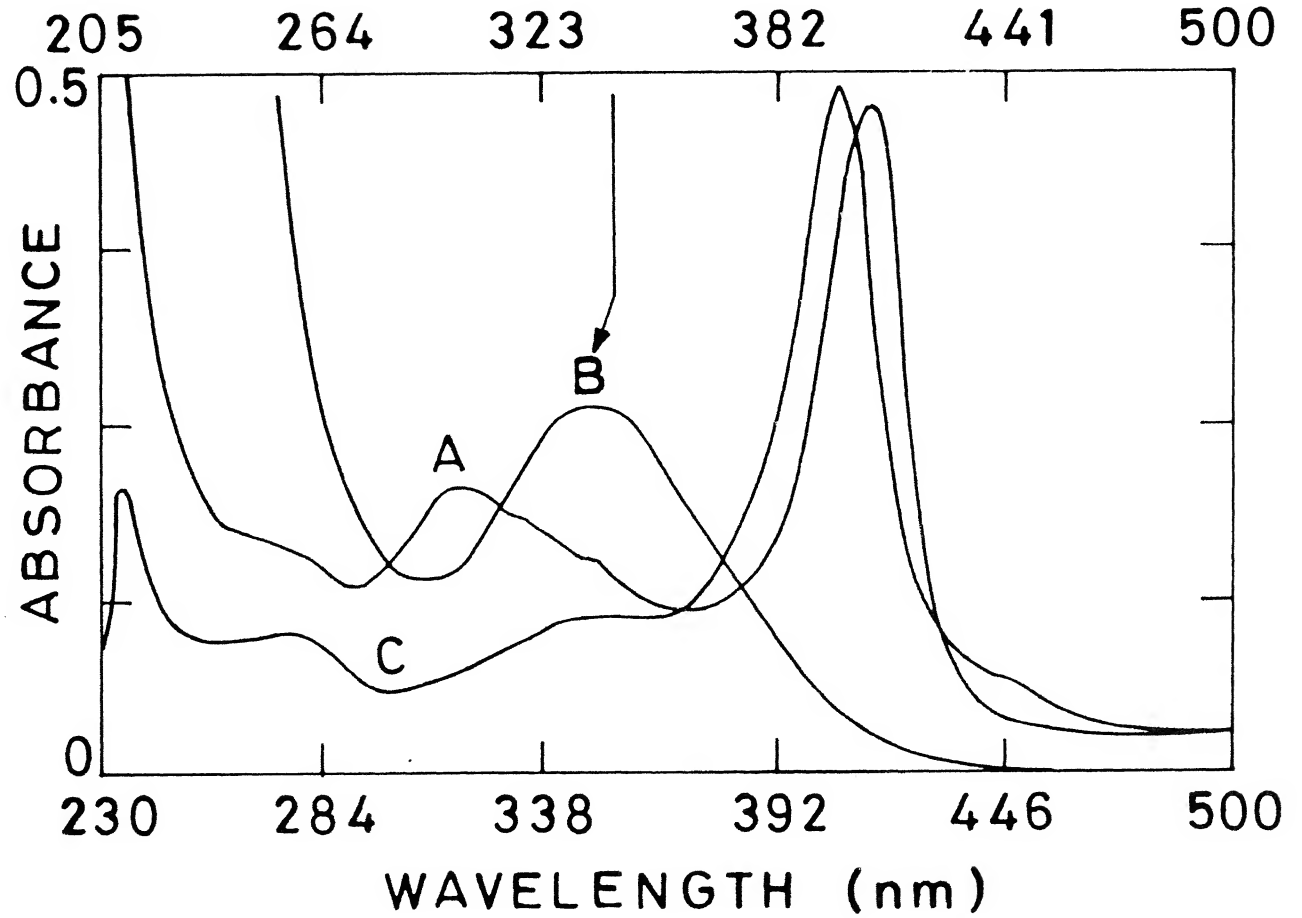


Fig. II.16(A). Evidence for separation of cytochrome-C and lipoic acid based on the absorption spectra of (A) binary mixture containing aqueous feed. (B) lipoic acid retained in aqueous extract after the contact with 50 mM AOT/isooctane. (C) cytochrome-C transferred in micellar phase from lipoic acid containing aqueous feed. Conditions are [cytochrome-C] = 0.5 mg/ml, [lipoic acid] = 0.5 mg/ml, 0.1M KCl, pH 10.

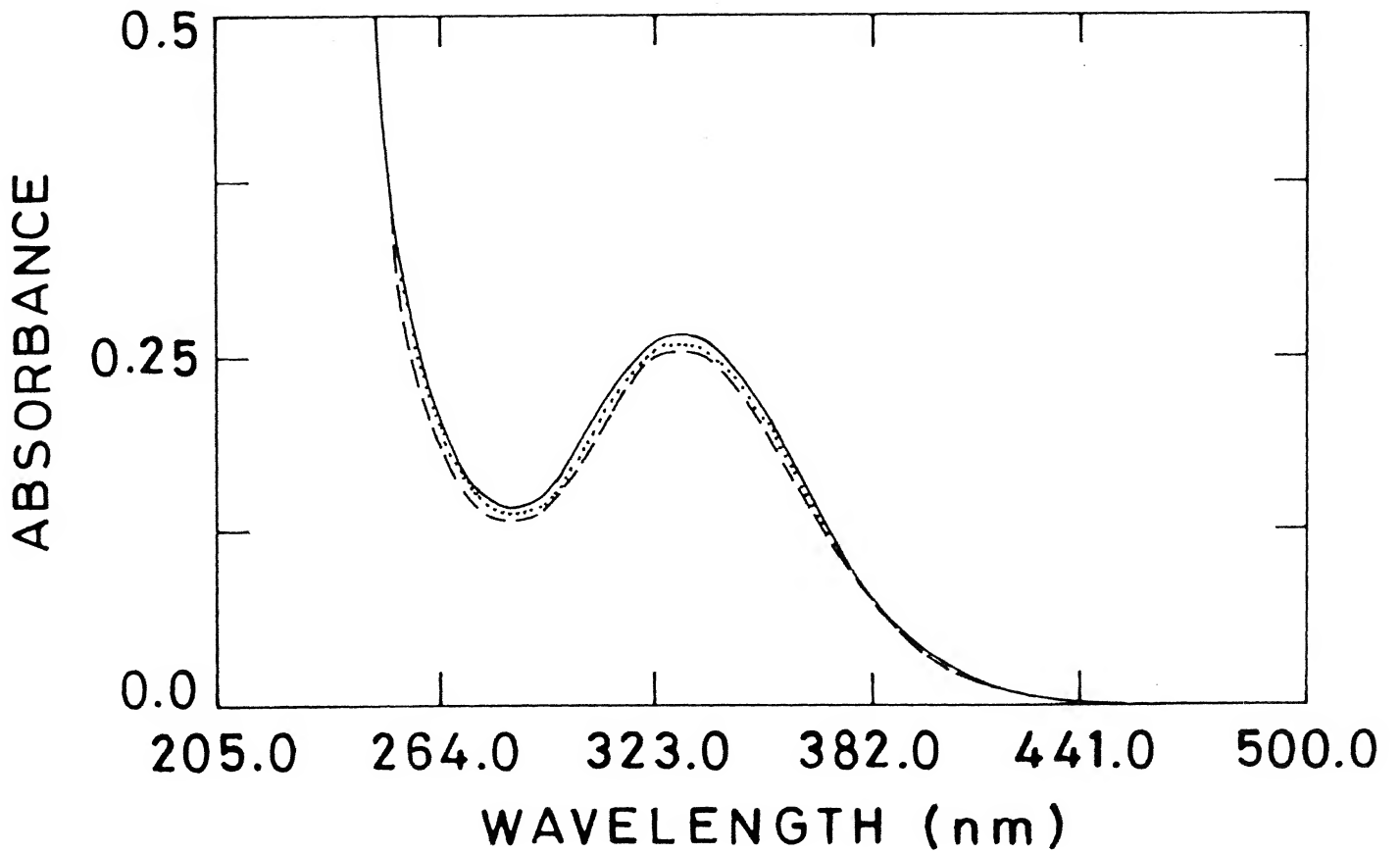


Fig. II.16(B). Absorption spectra of lipoic acid in different conditions of lipoic acid containing aqueous feed (——) in water, (---) retained in aqueous extract after the contact with 50 mM AOT/iso-octane (....) in aqueous extract retained after the separation from cytochrome-C on contact of (lipoic acids cytochrome-C) containing aqueous feed with 50 mM AOT/isooctane. Conditions are same as in Fig. 16(A).

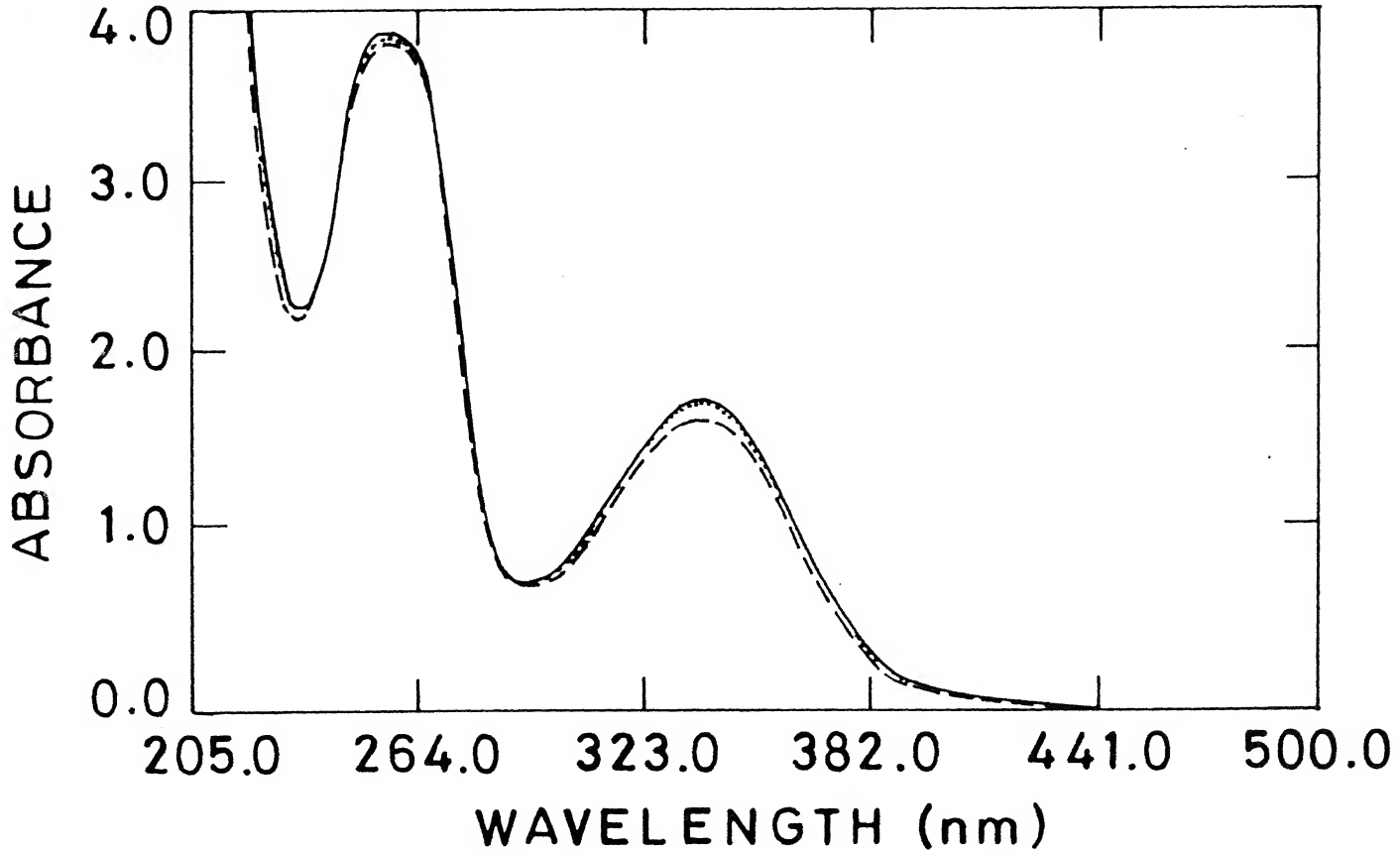


Fig. II.17. Absorption spectra of NADH in different conditions: (——) in water, 0.1M KCl, pH 7.0; (---) retained in aqueous extract on contact of NADH containing aqueous feed with 50 mM AOT/isooctane; (.....) retained in aqueous extract after the separation from creatine kinase (transferred in micellar phase). Conditions are: [creatine kinase] = 0.5 mg/ml, [NADH] = 0.5 mg/ml, 0.1M KCl, pH 7.0 on contact of mixture (creatine kinase & NADH) containing aqueous feed with 50 mM AOT/isooctane.

latively slow [12].

Similar experimental condition of pH and ionic strength etc. re adopted for the separation of cytochrome-C and lipoic acid. g. II.16(A) represents the absorption spectra which demonstrate ıe verification for the separation of cytochrome-C and lipoic :id in forward transfer. A is the absorption spectra of the .nary mixture while B and C show the characteristic spectra of .poic acid (λ_{max} 331 nm) and cytochrome-C (280 nm & 408 nm) :ter separation. Fig. II.16(B) is the absorption spectra of ipoic acid in different conditions mentioned in legend.

In similar way, creatine kinase has been separated from ADH. At pH 6.0, 0.15 M KCl containing aqueous feed of the ixture, creatine kinase is completely extracted from aqueous hase to reverse micellar phase, but NADH is retained in aqueous hase only. After contacting the protein containing micellar hase with 1.0 M KCl solution at pH 10.0, the creatine kinase is ack extracted from organic to aqueous phase. This process ompletes the separation of binary mixture very rapidly. The dentity of proteins and their quantity (concentration) after eparation from the mixtures, were determined by their haracteristic absorption spectra and A_{280} respectively. In the .V.-Vis. absorption spectra, two characteristic peaks of NADH ıre found at 260 nm & 340 nm but creatine kinase shows its ıaximum absorbance at 280 nm only. These studies are summarized .n Fig. II.17 which depicts the absorption spectra of NADH as ıuthentic sample and that of aqueous phase after forward transfer

of creatine kinase in 50 mM AOT/isooctane.

II.3.3.2 Separation of Protein from the Tertiary Mixture: Creatine Kinase + BSA + Lipoic acid

As BSA and lipoic acid do not solubilize at 0.15M KCl, pH 7.0, while the other protein creatine kinase almost completely solubilizes in 50 mM AOT/isooctane, the aqueous feed of protein mixture was adjusted to this pH and 0.15M KCl. On contacting this aqueous feed with the micellar phase, almost complete solubilization of creatine kinase occurs while all BSA and lipoic acid are retained in aqueous raffinate. The organic phase loaded with creatine kinase was back transferred to fresh aqueous solution of 1.0 M KCl at pH 10.0. BSA and lipoic acid containing aqueous phase was readjusted to pH 3.0 and then contacted second time with 50 mM AOT/isooctane. As BSA does not solubilize at any pH, it remains in the aqueous phase while lipoic acid completely solubilizes at pH 3.0 in micellar solution. Lipoic acid loaded in organic phase was recovered by back transfer into aqueous phase at 2.0 M KCl, pH 10.0. Spectral study of lipoic acid extracted in different way has been shown in Fig. II.16(B).

Cytochrome-C + Creatine Kinase + Lipase

Another set of tertiary mixture of cytochrome-C, creatine kinase and lipase was separated into single components and the proteins were recovered almost quantitatively by the above methods by monitoring pH and ionic strength of aqueous phase.

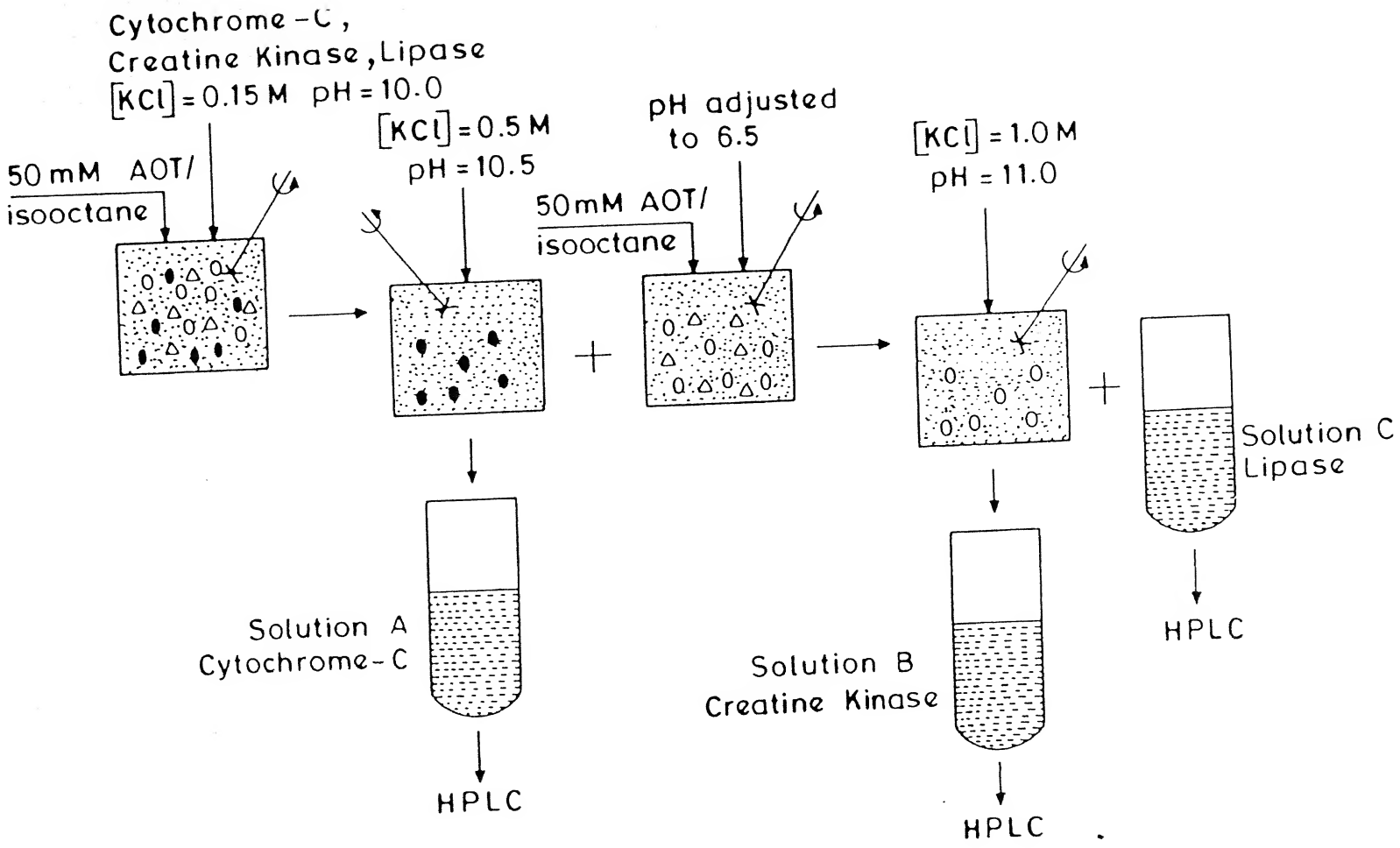


Fig. II.18. Representation of experimental procedure for the separation of proteins of a ternary mixture of cytochrome-C, creatine kinase and lipase.

ig. II.18 presents a simplified experimental procedure for solation of this ternary mixture. The aqueous solution ontaining proteins mixture was adjusted to pH 10.0, 0.15M KCl. his aqueous feed was contacted with 50 mM AOT in isooctane rganic phase. At pH 10.0, cytochrome-C completely solubilizes n the micellar phase while other two enzymes, creatine kinase nd lipase do not. The cytochrome-C was transferred from organic o aqueous phase according to the method described earlier. Now, he creatine kinase and lipase containing aqueous extract was djusted to pH 7.0. This raffinate was again contacted with 50 mM AOT/iso-octane. Lipase does not solubilize into micellar solution at this pH while creatine kinase readily solubilizes. This last process was repeated twice with fresh micellar solution in order to achieve complete separation of creatine kinase from lipase. Finally the creatine kinase loaded organic phase was contacted with 1.0M KCl solution at pH 10.0. In this way the enzyme was transferred from micellar phase back to aqueous phase.

All the proteins and biomolecules separated from the binary or ternary mixtures were estimated by absorbance at 280 nm and by Lowry method. The identification of single components transferred in forward and backward transfer was carried out by their characteristic u.v.-vis spectra. The separation of creatine kinase and lipase was also examined by using 10% SDS polyacrylamide gel electrophoresis.

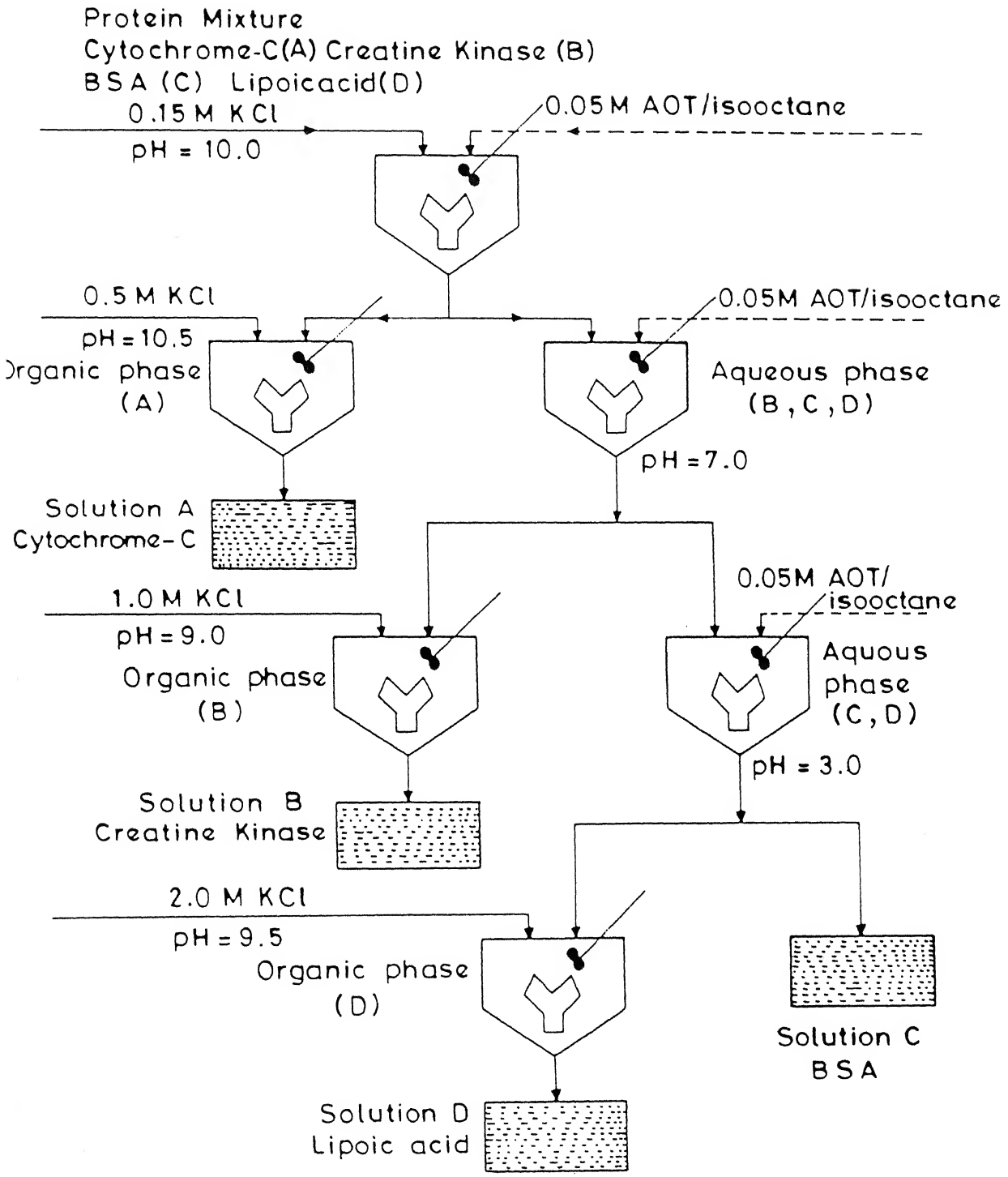


Fig. II.19. A simple representation of experimental flow chart for the separation of a quarternary mixture of cytochrome-C (A), creatine kinase (B), BSA (C) and lipoic acid (D) by using AOT/isooctane solution.

.3.3.3 Isolation of a Quarternary Mixture

A comparatively more difficult separation was achieved from ıe quarternary mixture of 0.25 mg/ml concentration of each of 'tochrome-C, creatine kinase, BSA and lipoic acid as shown by cperimental flow chart in Fig. II.19. In this case both the size xclusion factor and differences in the electrostatic ıteractions of the protein with the micelle, as controlled by pH nd ionic strength, were exploited to separate this mixture of ifferent type of biomolecules (namely enzymes/proteins and rowth factor).

Two ml of aqueous solution of quarternary protein mixture 'as adjusted to 0.15 M KCl, pH 10.0. In the first contact of this ıqueous feed with 2 ml of 50 mM AOT/isooctane solution followed ›y vortexing for 10 min. on a cyclomixture (~300 r.p.m.) and :entrifugation at 2000 r.p.m. for 20 min. at ~30°C, the two ıistinct phases were obtained. Careful separation of both the phases resulted in the extraction of cytochrome-C in organic phase and rest three components in the aqueous raffinate. Cytochrome-C was retransferred to aqueous solution with the process described in last section. The raffinate was now adjusted to pH 7.0 and contacted with fresh micellar solution of equal amount. This time, creatine kinase completely solubilized in the organic phase while BSA and lipoic acid were retained in the aqueous phase. Now, the binary mixture containing raffinate adjusted to pH 3.0 was contacted for the third time with fresh

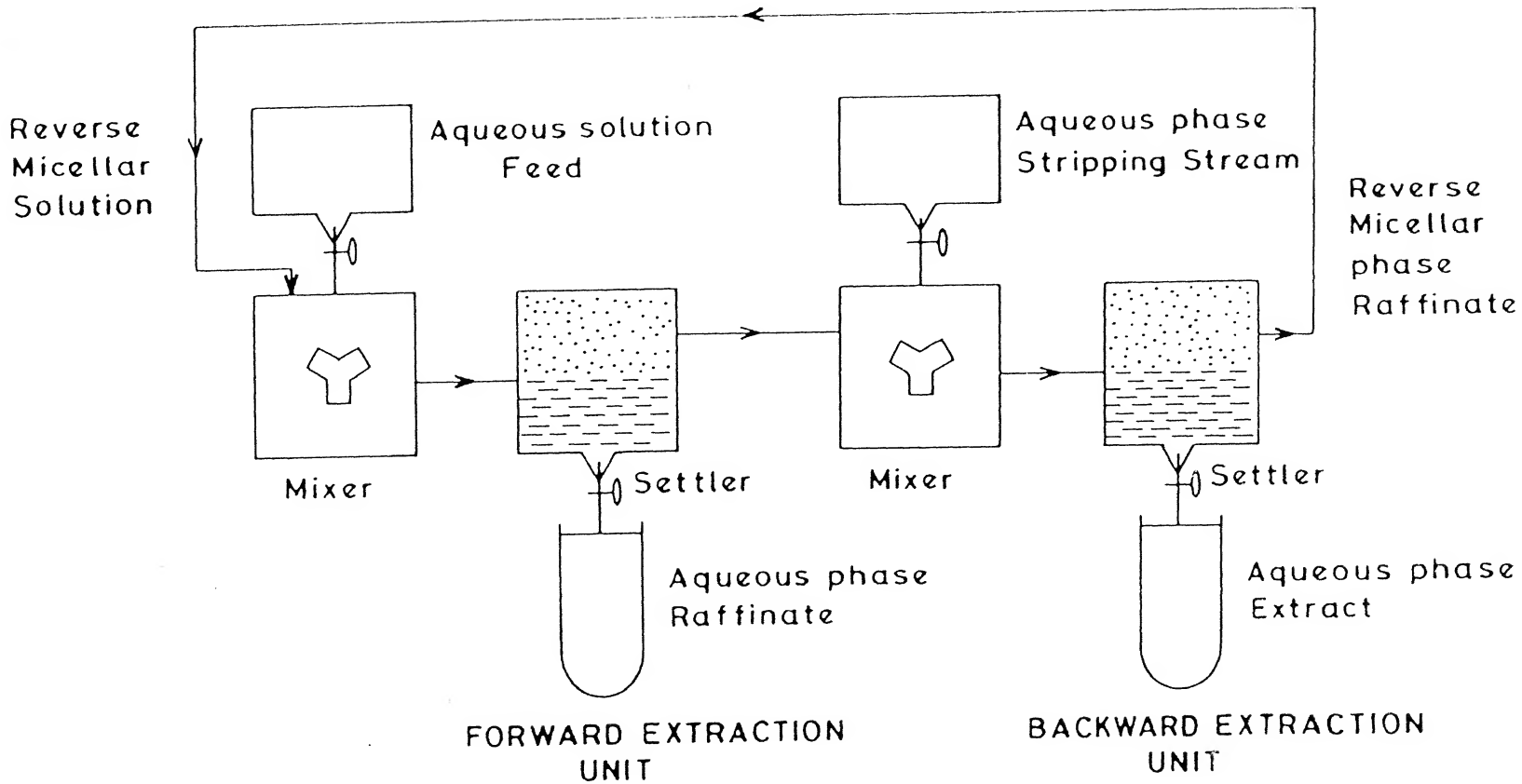


Fig. II.20. Schematic diagram of the continuous extraction system of the combined forward and back extraction of protein components for two mixer/setter units, with the reverse micellar phase circulating between the two extraction units.

amount of 50 mM AOT/isooctane. Lipoic acid easily migrated to the micellar phase under this condition whereas BSA remained as such in aqueous phase. In the last, creatine kinase and lipoic acid were recovered back to aqueous solution after their contact with equivolume of 1.0M KCl, pH 10.0 and 2.0M KCl, pH 10.0 respectively. The identification and determination of the proteins and lipoic acid were done by their respective value of A_{280} and U.V.-Vis. absorption spectra. Based on the principle of forward and backward extraction of proteins from aqueous phase to reverse micellar phase and vice versa and different investigations carried out by us and other workers on protein separations, the method can be scaled up to design a continuous extraction unit that extracts and concentrates enzymes/proteins and other biomolecules on line from a fermentation broth. Fig. II.20 is the simple representation of a continuous large scale double extraction process capable of transporting and concentrating the biopolymer, which is based on the flow sheet-diagram suggested by Dekker et al. [27]. In summary it appears that differences in protein size, charge and surfactant concentration, concentration of salt ions etc. can be employed to attain selective solubilization and thus separation of proteins. Future investigations will focus on other factors which also may play important roles in the recovery of proteins and bioproducts from different complex mixtures or fermentation broth.

II.4 Conclusion

From the experimental observations, it appears that both the liquid-liquid phase transfer process and extraction from the solid state are quite sensitive to characteristics of proteins and external parameters (like charge - pH functionality, concentrations of salt and surfactants, nature of organic solvents etc.) which govern different type of interactions between proteins and micelles. The results demonstrate the efficacy of the process in resolving the protein mixtures by using the reverse micellar solutions by manipulating the aqueous phase pH and ionic strength in accordance with the solubilization behavior exhibited by the individual proteins in a separate series of experiments. The results suggest that even very complex mixtures can be resolved and desired proteins or biomolecules may be recovered in their active form. These examples, though limited in scope, focus on the feasibility of bioseparation with reverse micelles for some systems. Thus selective and controlled solubilization of proteins and other biomolecules in reverse micellar organic phase is a promising technique that has the potential to be developed into a liquid-liquid extraction technology for the application in efficient recovery and concentration of selected proteins from fermentation broth and cell culture media.

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CHAPTER III

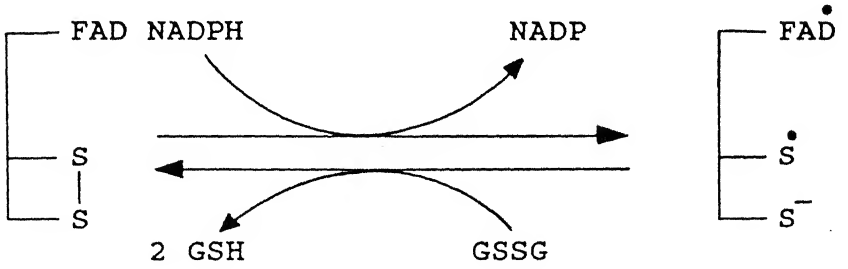
ACTIVITY AND KINETIC CHARACTERISTICS OF GLUTATHIONE REDUCTASE IN VITRO IN REVERSE MICELLAR WATERPOOL

[II.1 Introduction

Traditionally, enzymes, the natural catalysts of biochemical processes, have evolved so as to exhibit their maximal catalytic activity in aqueous medium. Temptation for the industrial use of enzymes due to their specificity and enormous catalytic activity is gradually increasing for the production of valuable biologically active materials and other important compounds. The application of enzymes as catalysts in fine organic synthesis and in the production of pharmaceuticals etc. has been referred to earlier (chapter I). However, wider use of enzymes is restricted due to instability and inactivation of enzymes in non-aqueous solvents. Unfortunately the transfer of an enzyme from water as reaction medium to an organic solvent is accompanied either by the complete denaturation of the enzyme or by a marked fall in its catalytic activity and the disappearance of its substrate specificity [1]. The present work describes a method for utilizing enzymes as catalysts in organic solvents through their encapsulation within reverse micelles. The retention of enzyme

ıctivity in reverse micellar system in organic solvents has made :his medium viable alternative medium for the study of enzymes in vitro [2-12]. Nevertheless, there is a need to study the ɔehavior of different enzymes in reverse micelles, in order to ınderstand the characteristics of enzymes in this novel mdeia.

In this chapter, a detailed and systematic study on the activity, stability and kinetic characteristics of glutathione reductase from two sources (Yeast and Bovine Intestinal Mucosa) has been reported in the solvent $CHCl_3$- isooctane (1:1, v/v) in presence of surfactant cetyltrimethylammonium bromide (CTAB) and a small amount of water. The enzyme glutathione reductase (NADPH: Oxidized glutathione oxidoreductase, EC 1.6.4.2) from both sources catalyses the NADPH dependent reduction of the disulfide bond of oxidized glutathione. It is a dimer with one FAD per monomer of 50 k dalton. The scheme of the catalysis is shown below [13].



Scheme III.1

In living system, the wide functional distribution of reduced glutathione [14] reflects the general importance of glutathione reductase. In particular, reduced glutathione contributes to the

.ability of the erythrocyte by stabilizing thiols in the cell embrane, in hemoglobin, and in cellular enzymes [14,15]. Glutaıione reductase has attracted attention as a representative xample of flavin enzymes, and much of the work on glutathione eductase has been carried out on the enzyme from yeast[13,16-17] n aqueous solution. However, the solubilization, stability and inetic investigations in reverse micelles in non-polar media, ave been studied for the first time by us [18].

The study of the reduction of disulfide bond presents a new :lass of investigation in [CTAB/H_2O/$CHCl_3$-isooctane] reverse nicelles. For simplicity, in the present report, we generally ıse the term 'reverse micelle' for the aggregation of surfactant monomers in organic solvents, however, the term water-in-oil microemulsion is more appropriate at large W_o. The waterpool of the reverse micelle is expressed by the molar ratio W_o = [H_2O]/[CTAB].

III.2 Experimental Section

III.2.1 Materials

Glutathione reductases (EC 1.6.4.2) from yeast and bovine intestinal mucosa as the crystalline suspension were purchased from Sigma Chemical Co. St. Louis U.S.A. The coenzyme NADPH was also obtained from Sigma Chemical Co. Oxidized glutathione (GSSG) was procured from Boehringer-Mannheim, F.R.G. The surfactants CTAB (cetyltrimethylammonium bromide) and cetrimide (mixed alkyltrimethylammonium bromide) & TDTAB (tetradecyltri-

methylammonium bromide) were obtained from SRL, Bombay, India and Sigma Chemical Co. U.S.A. respectively. These surfactants were used as such after drying over P_2O_5 for several hours in a vacuum desiccator. Isooctane, puriss grade was from Fluka, Switzerland and chloroform, AR was from BDH, U.K. All the solvents were purified before use by standard methods. Buffer components like potassium phosphates, trizma base, glycine EDTA and β-mercapto-ethanol were also obtained from Sigma. All other chemicals were of analytical grade. Double distilled water was used for all the aqueous solutions.

III.2.2 Methods

III.2.2.1 Enzyme Purification

Gel filteration chromatography on a 1 x 30 cm. Sephadex G-25 column at $4^{o}C$ was used to remove ammonium sulfate from the enzyme preparation of glutathione reductase. Purity/homogeneity of the enzyme was checked by SDS gel electrophoresis on 8% acrylamide gel using the method of Laemmli [19].

III.2.2.2 Preparation of Reverse Micellar Solution Containing Enzyme and Substrates

The reverse micellar solution was prepared by dissolving the required amount of surfactant in the known amount of $CHCl_3$-isooctane (1:1, v/v) mixture. The solution is thermodynamically stable and optically transparent at room temperature. Homogeneous (optically transparent, i.e. non turbid) solution of enzyme and

substrates in reverse micelles was obtained by injection method (cf. chapter I). The solubilization process is remarkably dependent on the parameters like water to surfactant molar ratio (W_o), pH and ionic strength of the buffer solution, surfactant concentration and temperature etc.

In a typical experiment, reverse micelles containing the desired concentration of substrates and enzyme were generated by injecting, by microsyringe, required volumes (4 μl) of aqueous solutions of oxidized glutathione (125 mM), NADPH (50 mM) and glutathione reductase (40 μg/ml) into the 2 ml solution of each of reverse micelles CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) and cetrimide/$CHCl_3$-isooctane. The waterpool size of the reverse micelles was adjusted by injecting additional volumes of aliquots of 0.1 M potassium phosphate, 1 mM EDTA, 1 mM β-mercaptoethanol buffer solutions at different pH (pH 6.0-9.0). The reaction mixture was agitated on a vortex mixer for a few seconds for solubilization of aqueous solution into reverse micelles.

III.2.2.3 Enzyme Activity Measurement

The assay of glutathione reductase was done by recording the decrease in absorbance of NADPH at 340 nm on a Gilford Response Spectrophotometer at 30$\pm$0.1^{o}C. The temperature of the cell was maintained by circulation of water in the cuvette holder from an external thermostat equipped with a high precision electronic relay. Freshly prepared micellar solution was taken in the reference cell, and the substrates and enzyme containing reverse

micellar solution was transferred in the sample cell. The kinetic run was monitored after addition of aqueous NADPH solution in the sample micellar solution. Adequate controls were run to ensure that the decrease in absorbance represented the enzyme activity. Molar absorption coefficient for NADPH was taken as 6.22×10^3 $M^{-1}cm^{-1}$ in reverse micellar and in aqueous systems.

The specific activity of glutathione reductase in aqueous buffer was 136.5 units/mg for yeast source and 65 units/mg for bovine intestinal mucosa source. (One unit reduces 1.0 μmole of oxidized glutathione per min. at pH 7.5 at 30°C). The calculation of the specific activity of the enzyme was performed as below.

III.2.2.4 Calculation of Specific Activity of Enzyme

The specific activity of the enzyme is expressed as unit/mg of protein. One unit of enzyme is defined as the amount of enzyme that will form one μmole of $NADP^+$ per minute.

By Beer-Lambert Law:

$$\text{Absorbance, } A = ECl \text{ or } C = \frac{A}{El}$$

where E = extinction coefficient

C = concentration in moles/litre

l = path length

Here, $E^{NADPH} = 6.2 \times 10^3$ litre $mole^{-1}cm^{-1}$ at 340 nm.

& l = 1 cm.

then $$C = \frac{A_{340}/min}{6.2 \times 10^3 \times 1} \text{ (mole/litre) min}^{-1}$$

or $$C = \frac{A_{340}/min}{6.2} \text{ (}\mu\text{mole/ml) min}^{-1}$$

Hence, specific activity = $$\frac{A_{340}/min}{6.2 \times \text{mg enzyme/ml reaction mixture}}$$

------ III.1

III.2.2.5 Absorption Spectra

U.V.-Vis absorption spectra were recorded with Gilford Response spectrophotometer, using 1 cm. quartz cells at 30°C. The concentration of NADPH was determined from the extinction coefficient at pH 7.0 of 6.22×10^3 $cm^{-1}M^{-1}$ at 340 nm. The same extinction coefficient was used for the corresponding micellar solution. Enzyme concentrations were checked by Lowry method.

III.3 RESULTS AND DISCUSSION

III.3.1 Study of Glutathione Reductase from Yeast

The reverse micellar solution of CTAB in $CHCl_3$-isooctane (1:1, v/v) was found to be quite suitable for solubilization of glutathione reductase from yeast. This hydrophilic enzyme after entrapment inside the reverse micelles, provides a new milieu for the study of enzymatic behavior. It is of interest to note that the concentrations of the solubilized enzymes/hydrophilic species can be expressed in two ways. One is relative to the volume of

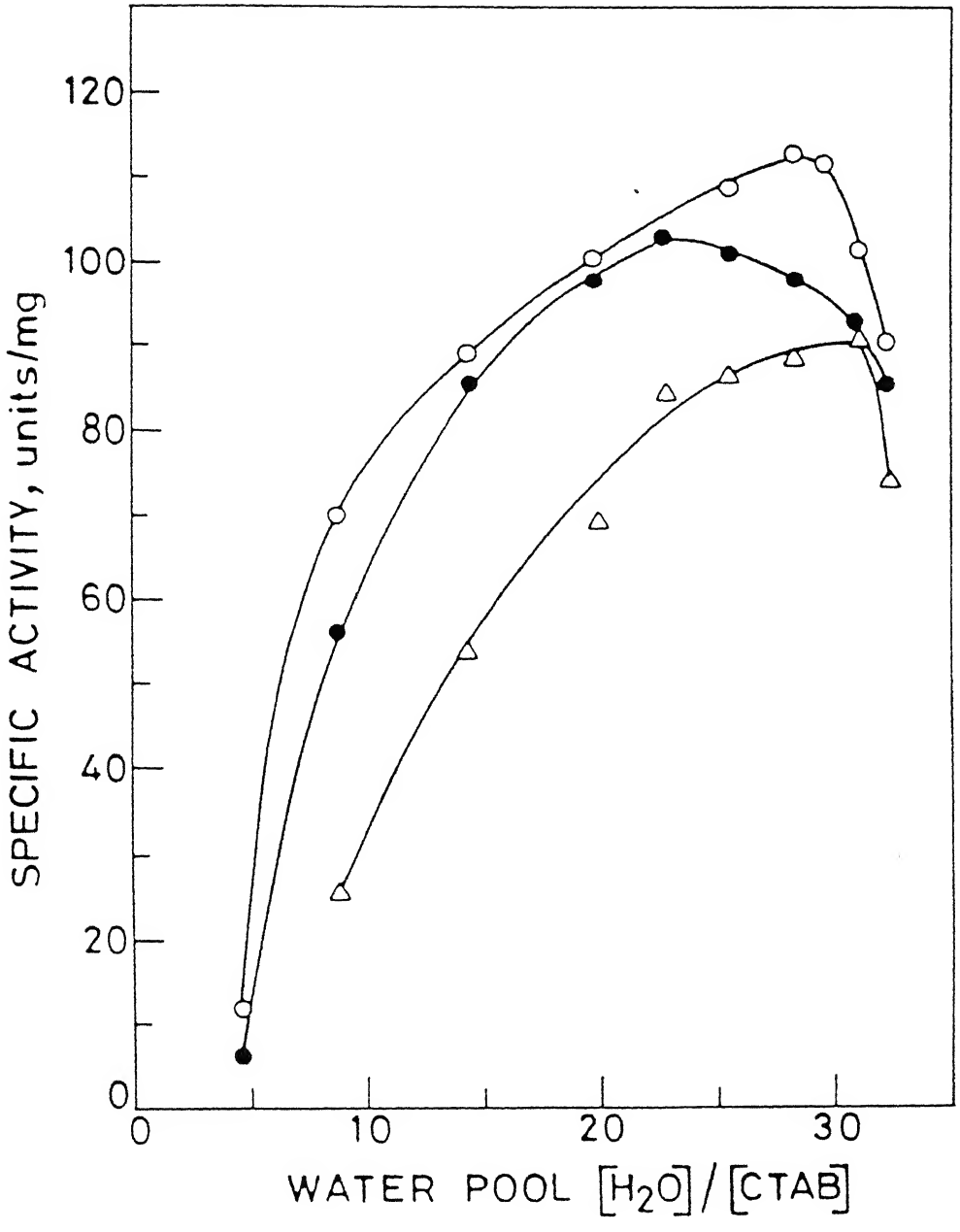


Fig. III.1. Activity of glutathione reductase in reverse micelles, 0.1M CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) as a function of W_o at different pH (△, pH 7.25; O, pH 8,25; ●, pH 8.50 of 0.1 M potassium phosphate, 1 mM EDTA), [E] = 0.08 μg/ml, [GSSG] = 250μM; [NADPH] = 100μM.

waterpool, where reaction takes place and the other is with respect to the overall solution volume (water + organic solvent). For a complete hydrophilic compound, like an enzyme or other hydrophilic substrates, the two concentrations can be correlated, as follows [20].

$$C_{ov} = C_{wp} \cdot F_W \qquad \text{-----III.2}$$

Where F_W is the percent water and C_{ov} and C_{wp} are the overall concentration and local concentration (waterpool) respectively. In all the experiments, overall concentration has been considered unless stated otherwise.

In order to establish the conditions for the optimum activity of yeast glutathione reductase in CTAB/H_2O/$CHCl_3$-iso-octane system, the activity was determined at different pH, water content and surfactant concentrations etc. It was found that these parameters affected remarkably the behavior of enzyme in this reverse micellar system.

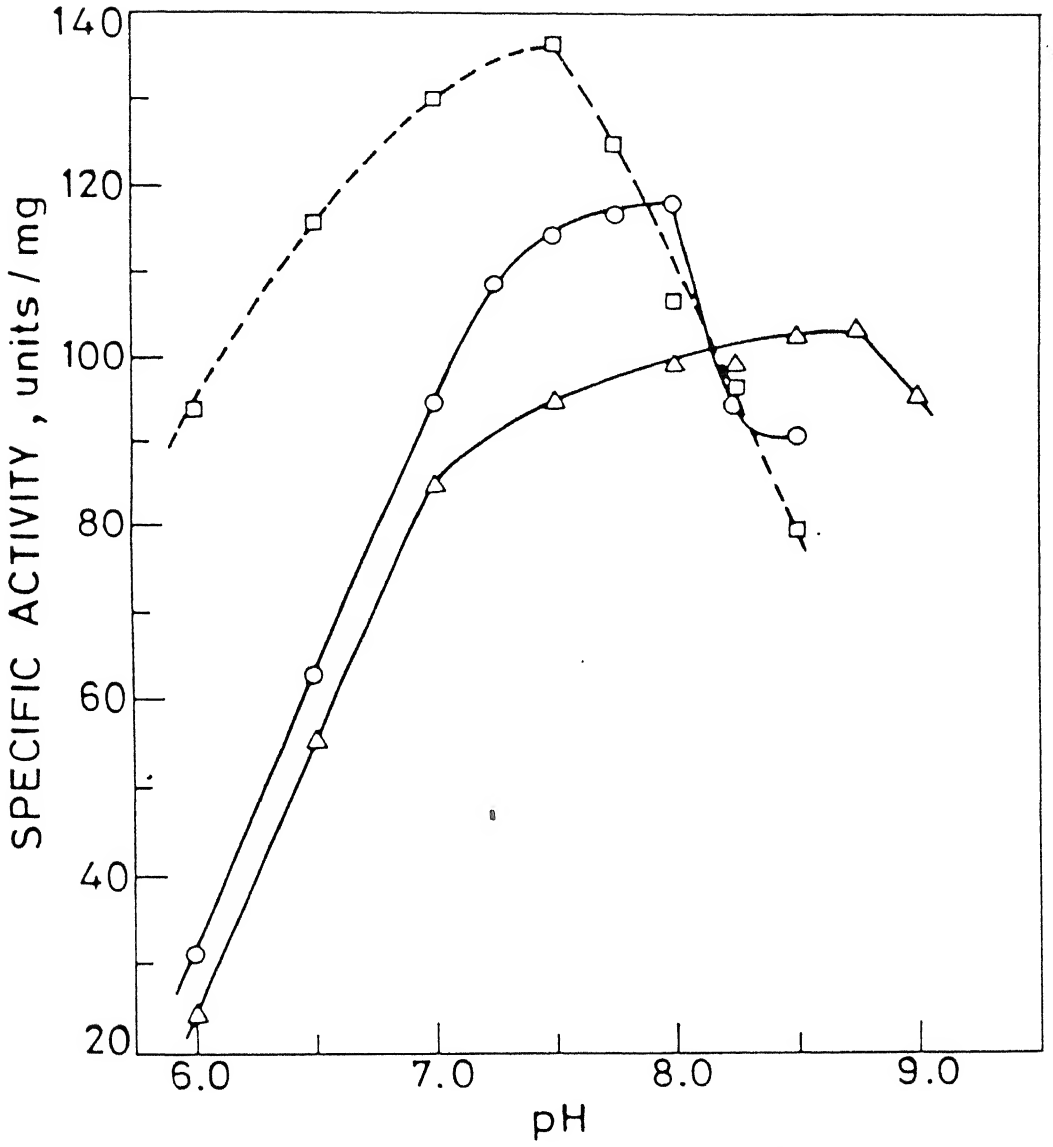
III.3.1.1 Effect of W_o on Activity

In the reverse micelle's polar core, the waterpool is the important region where the entrapped enzyme is protected from the unfavourable action of the organic solvents. The size of the waterpool may be varied by changing either the water content or the surfactant concentration. Fig. III.1 shows the variation in specific activity of glutathione reductase as a function of W_o at different pH values. These pH values represent pH of the stock

buffer solutions injected into CTAB/$CHCl_3$-isooctane (1:1, v/v) solution. At every pH, the enzyme showed a bell shaped curve with maximum activity towards the high value of W_o at low pH. This type of activity - W_o profile has also been found in the case of α-chymotrypsin [21] and recently studied enzymes malate dehydrogenase [7], lactate dehydrogenase [8], glucose-6-phosphate dehydrogenase [22] and dihydrofolate reductase [10]. It appears that the bell shaped dependence of activity upon the magnitude of W_o represents a general trend in micellar enzymology. Glutathione reductase in reverse micelles shows maximum activity towards the higher W_o values. Few enzymes in the AOT/isooctane system show the optimal activity at the lower W_o values in the range 8-12. For example, maximal activity of α-chymotrypsin [21] was found to be at W_o = 7. Lysozyme [23] and lipase [24] exhibited the highest activity at W_o values of 8 and 10, respectively. However, in the CTAB, cationic reverse micelles, the maximal activity of lysozyme [25] and trypsin [26] was found at W_o = 20.3, pH 7.0 and W_o = 25, pH 8.0 respectively. At this stage, the reason for the highest activity of many enzymes at higher W_o in cationic reverse micelles of CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) and at lower W_o in anionic reverse micelles of AOT/isooctane is not clear.

III.3.1.2 Effect of pH on Activity

The variation of enzyme activity as a function of pH at two different W_o values is given in Fig. III.2. It is of interest to



III.2. Activity of glutathione reductase in water (-□-□-) and in reverse micelles, 0.1M CTAB/H_2O/$CHCl_3$-iso-octane (1:1, v/v) as a function of pH at different W_o values (o——o, W_o = 29.7; △-△, W_o = 20.0). [E] = 0.08 μg/ml, [GSSG] = 250 μM; [NADPH] = 100 μM. Buffer used was 0.1M potassium phosphate, 1 mM EDTA.

note that a bell shaped curve was obtained at every W_o. The pH profile of glutathione reductase is identical in nature to the results obtained in aqueous media. Maximum activity was shown by the enzyme towards the lower pH and higher W_o values. In this experiment, maximum activity of glutathione reductase was found at a value of W_o = 29.7 and pH 8.0. At pH > 8.0 the enzyme exhibited more activity in reverse micelles than that in water, at the corresponding pH value. However, at low pH the activity is lower than that in water. Furthermore, glutathione reductase shows a shift in optimum pH by 0.5 unit in comparison to the optimum pH in aqueous medium (Fig. III.2). The pH shift of the magnitude of 0 to 3 unit in transition from aqueous medium to reverse micellar medium has been observed in other system as well. However, Martinek et al. [26] in the case of trypsin in CTAB/$CHCl_3$/octane system and Fletcher et al. [9] for α-chymotrypsin in CTAB/$CHCl_3$/heptane system have reported no shift in optimum pH.

At low pH and low W_o the enzyme loses its activity. Only around 60% activity remained at pH 7.0. As the pH of the buffer solution is raised to 8.0, the enzyme exhibits approx. 90% activity (at opt. W_o = 29.7), suggesting thereby that glutathione reductase acquires its active conformation. A small fraction of loss in specific activity of the enzyme might arise due to the exposure of the enzyme to organic solvent while passing into the waterpool of reverse micelles. At W_o other than 29.7, the decrease in enzyme activity might be attributed to the change in

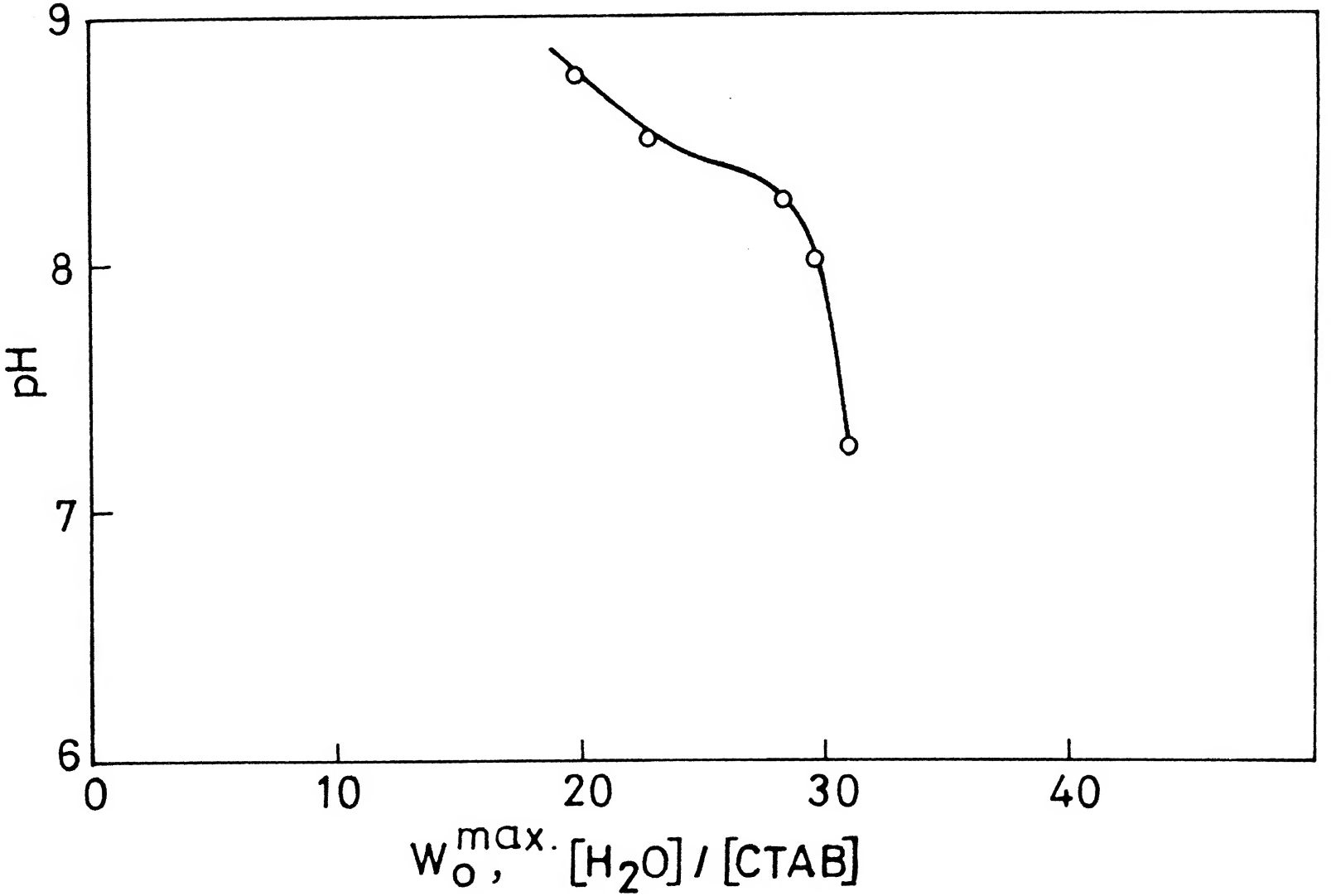


Fig. III.3. Dependence of $W_{o,opt.}$ on $pH_{opt.}$ of the aqueous buffer transferred in the reverse micelle, conditions are same as in Fig. 2 & Fig. 3.

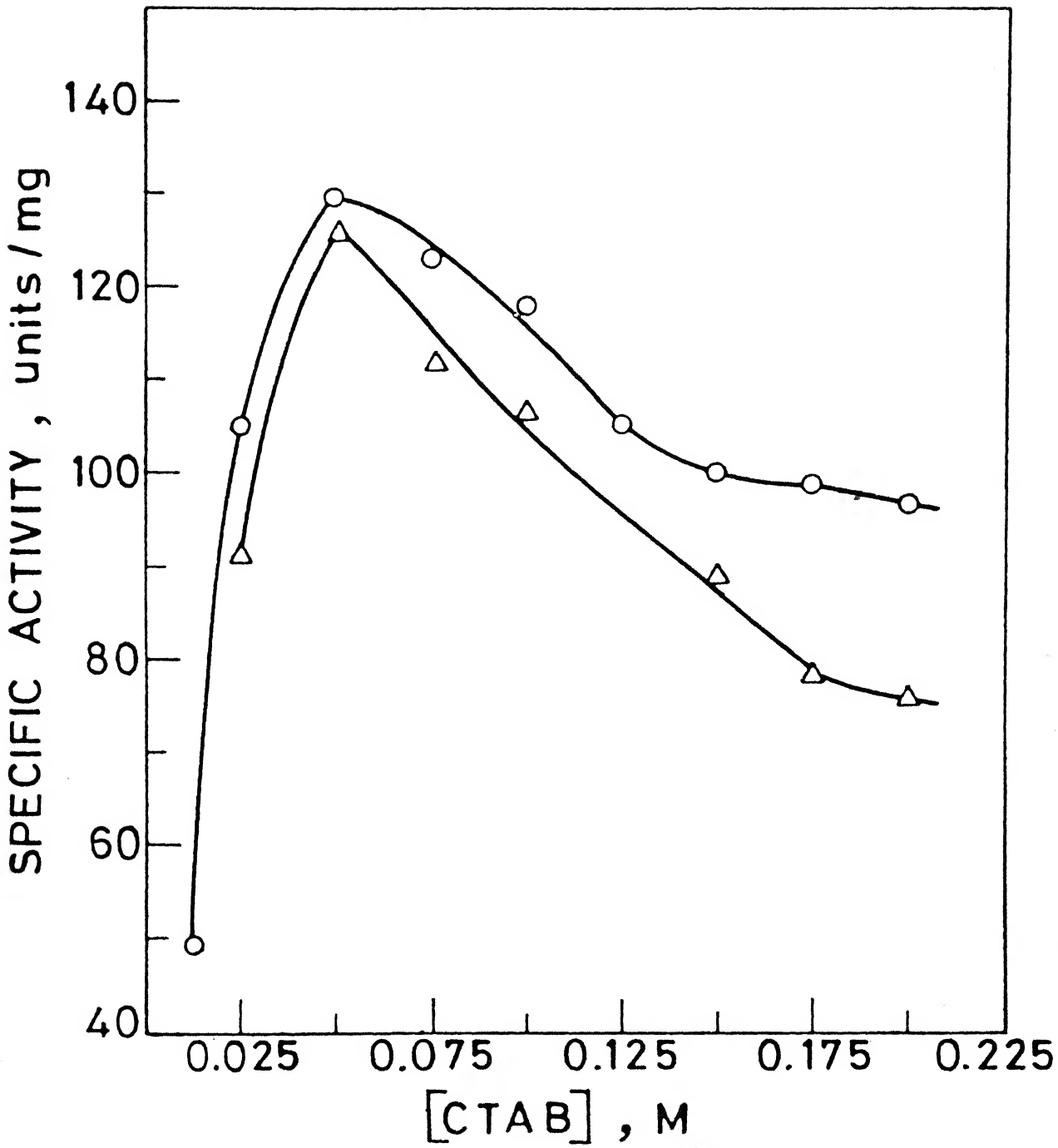


Fig. III.4. Dependence of the specific activity of glutathione reductase in reverse micelles at different concentrations of CTAB in $CHCl_3$/isooctane (1:1, v/v) at W_o = 29.7, pH 8.0 (O) and W_o = 20.0, pH 8.0 ( ). [NADPH] = 100 μM, [GSSG] = 250 μM, [E] = 0.08 μg/ml. Specific activity of glutathione reductase in 0.1M potassium phosphate, 1 mM EDTA (pH 7.5) is 136.5

been found to be less active. One of the probable reason for decrease in activity may be like this. With the increase in surfactant monomers, the number of micelles which host the enzyme and other substrates will increase. As a result of that mean free path for collision of two micelles decreases and the collision frequency (i.e. no. of collisions per second) increases and thus probability of fusion of two micelles enhances. In this process the contents of two micelles get more exposure to organic solvent resulting in the decrease of activity with increasing concentration of surfactant. At very low surfactant concentration, the aggregation of monomers might not be able to provide the protective compartment from organic solvent.

III.3.1.4 Kinetic Studies

Modeling of the kinetics of enzyme reactions within reverse micelles is complicated by the microscopically heterogeneous nature of the medium. One potential concern is that the transport of substrate and product into and out of the micelles may be the rate-limiting step. In the reverse micellar systems studied thus far, the reverse micelle concentration exceeds the enzyme concentration, implying that there will be both empty and filled micelles, and that the filled micelles will usually contain only one enzyme molecule. Reverse micelles continually collide, fuse and reform, exchanging their contents [27]. The characteristic time for the formation of the temporary dimer micelles is 10^{-6} to 10^{-10} s [28], much faster than the typical

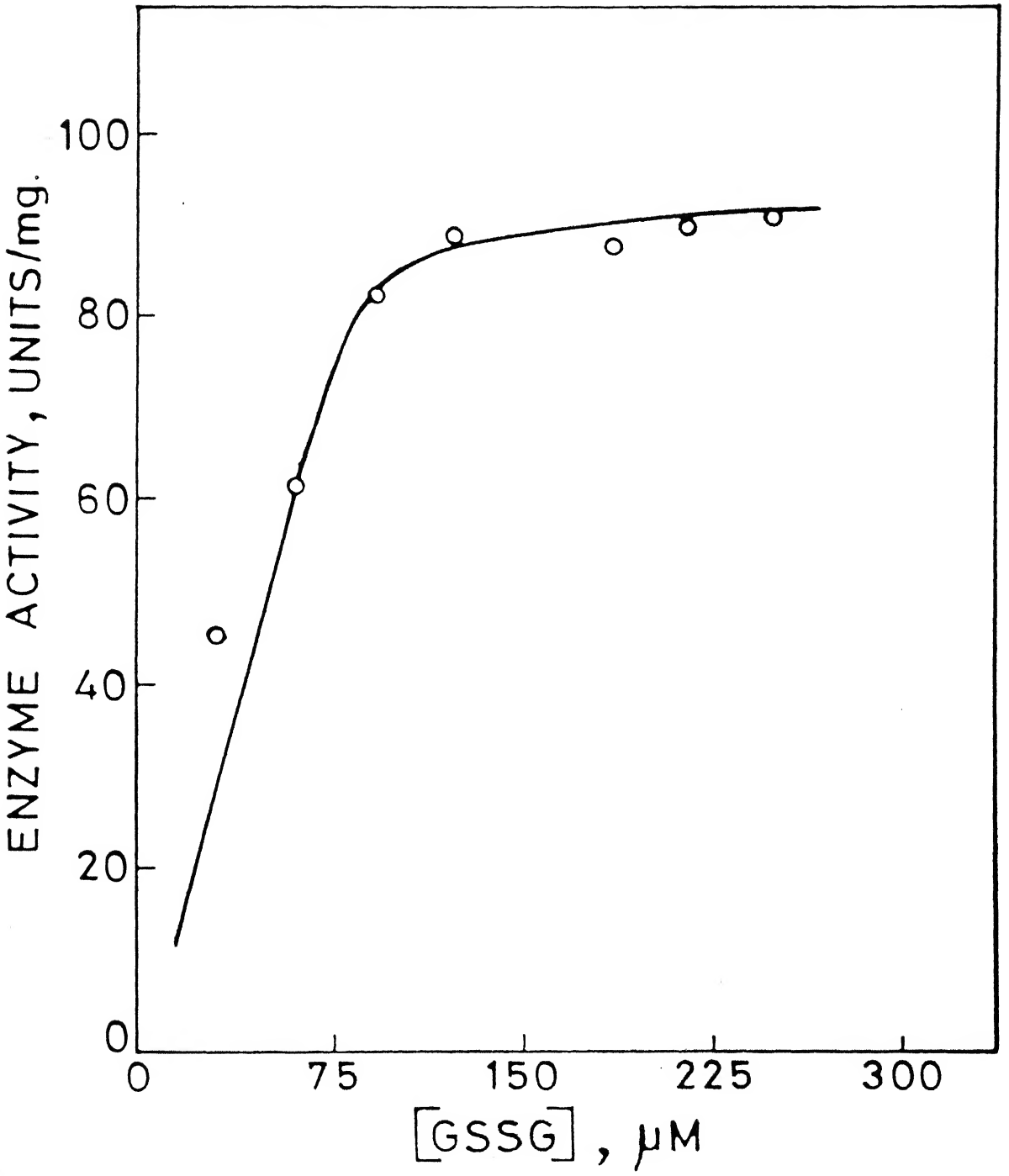


Fig.III.5(A). Dependence of glutathione reductase activity on the substrate concentrations in reverse micelles, 0.1 M CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) at W_o = 31.1, pH 7.25; oxidized glutathione [GSSG] at fixed [NADPH] = 100 μM, [E] = 0.08 μg/ml.

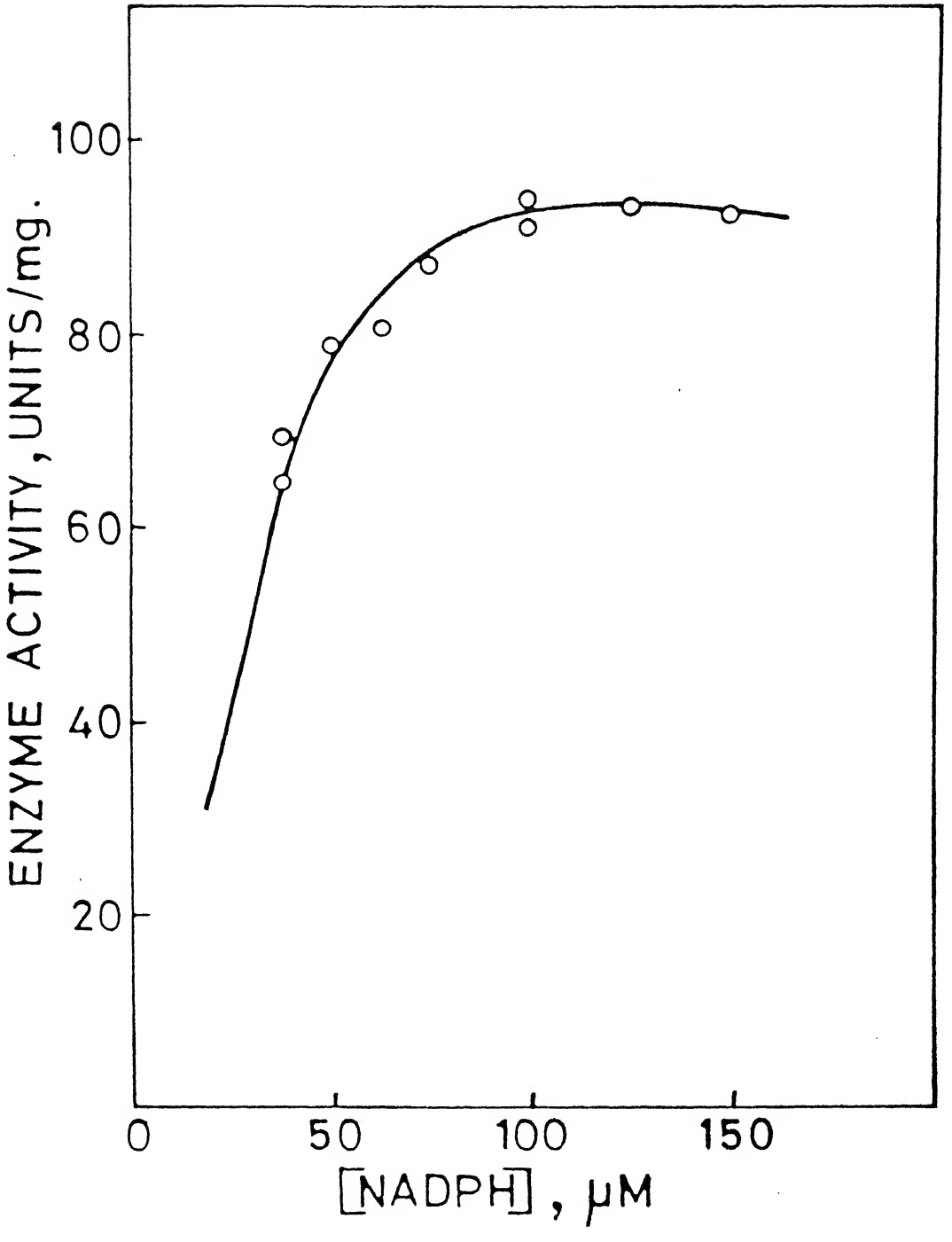


Fig.III.5(B). Dependence of glutathione reductase activity on the substrate concentrations in reverse micelles, 0.1 M CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) at W_o = 31.1, pH 7.25, [NADPH] at fixed [GSSG] = 250μM, [E] = 0.08 μg/ml. Buffer components are same as in other conditions.

characteristic time for enzymatic reactions, viz. $1/k_{cat} \sim 10^{-2}$ to 10^{-5}s [28]. If the number of unfilled micelles far exceeds the number of filled micelles, the enzyme experiences an effectively homogeneous aqueous environment. In such a case, any potential kinetic limitation due to transport resistance of substrate or product across the micelle surfactant boundary is reduced by the large total surface area per enzyme molecule and the rapid mixing of internal contents between micelles.

As the hydrophilic enzyme is considered to be active only in the aqueous pseudophase, the substrate and enzyme concentration within the reverse micelles are the important parameters in determining reaction kinetics. Yet, only the observed overall characteristics of the system are experimentally identifiable. Measured values and variables involving concentration can be referred to the overall, subscripted 'ov'. Levashov et al. [29] has developed Michaelis-Menten kinetics for an enzymatic reaction within reverse micelles .

III.3.1.4.1 Effect of Substrate Concentration

The activity of glutathione reductase in reverse micelles depends on the concentration of oxidized glutathione [GSSG] and [NADPH] solubilized inside the micellar core. Figs.III.5(A) and III.5(B) represent the variation of glutathione reductase activity as a function of concentration of [GSSG] and [NADPH] respectively. With the increase in substrate concentration the rate of the enzymatic reaction increases linearly in the beginning

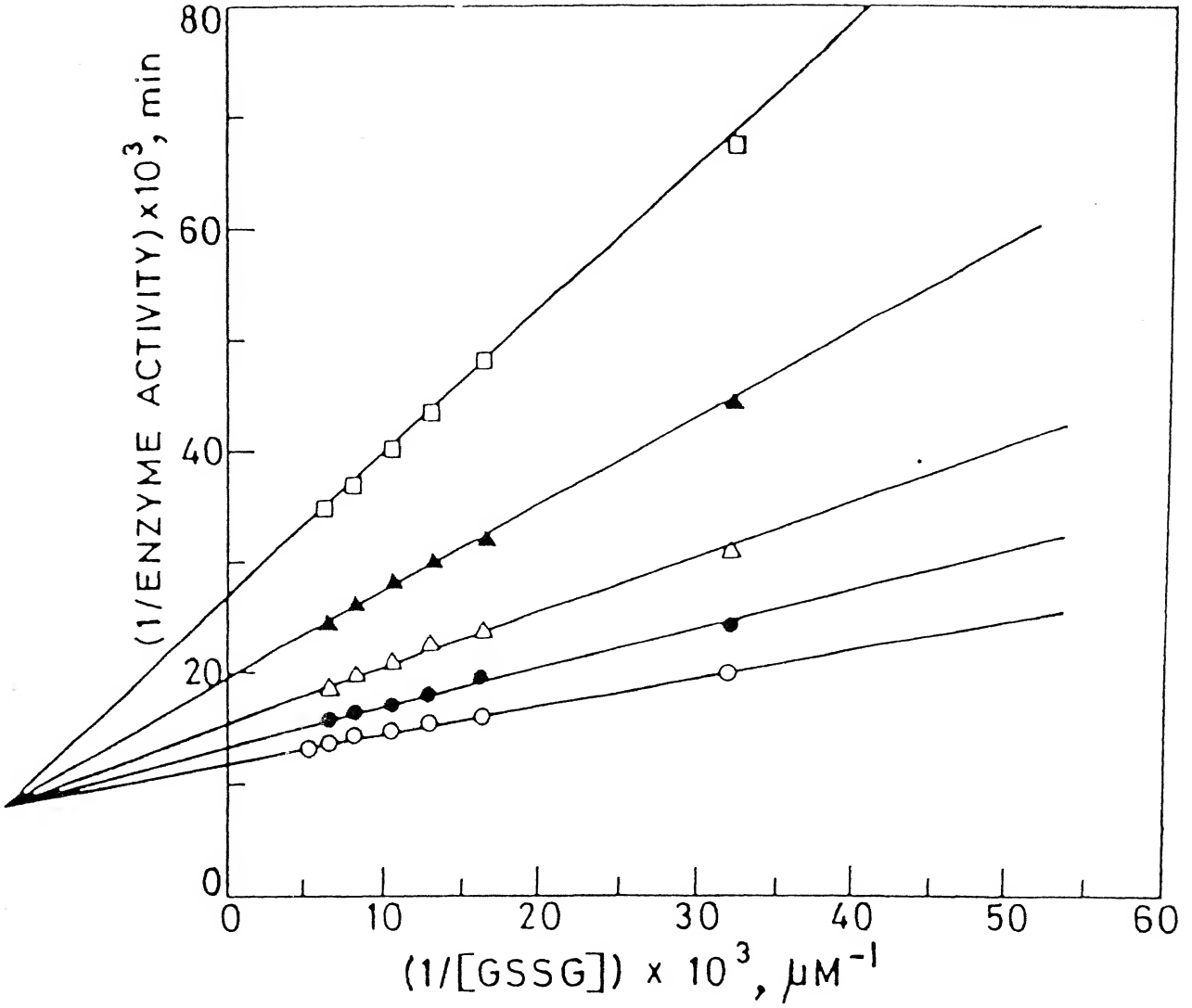


Fig. III.6. Lineweaver-Burk plots for the initial rate of glutathione reductase in 0.1M CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) with the varying substrate $[GSSG]^{-1}$ at W_o values of 29.7, pH 8.0 at fixed [NADPH]; NADPH concentrations were 12.5 μM (◇), 25 μM (▲), 37.5 μM (△), 50 μM (●), 75 μM (○), [E] = 0.08 μg/ml.

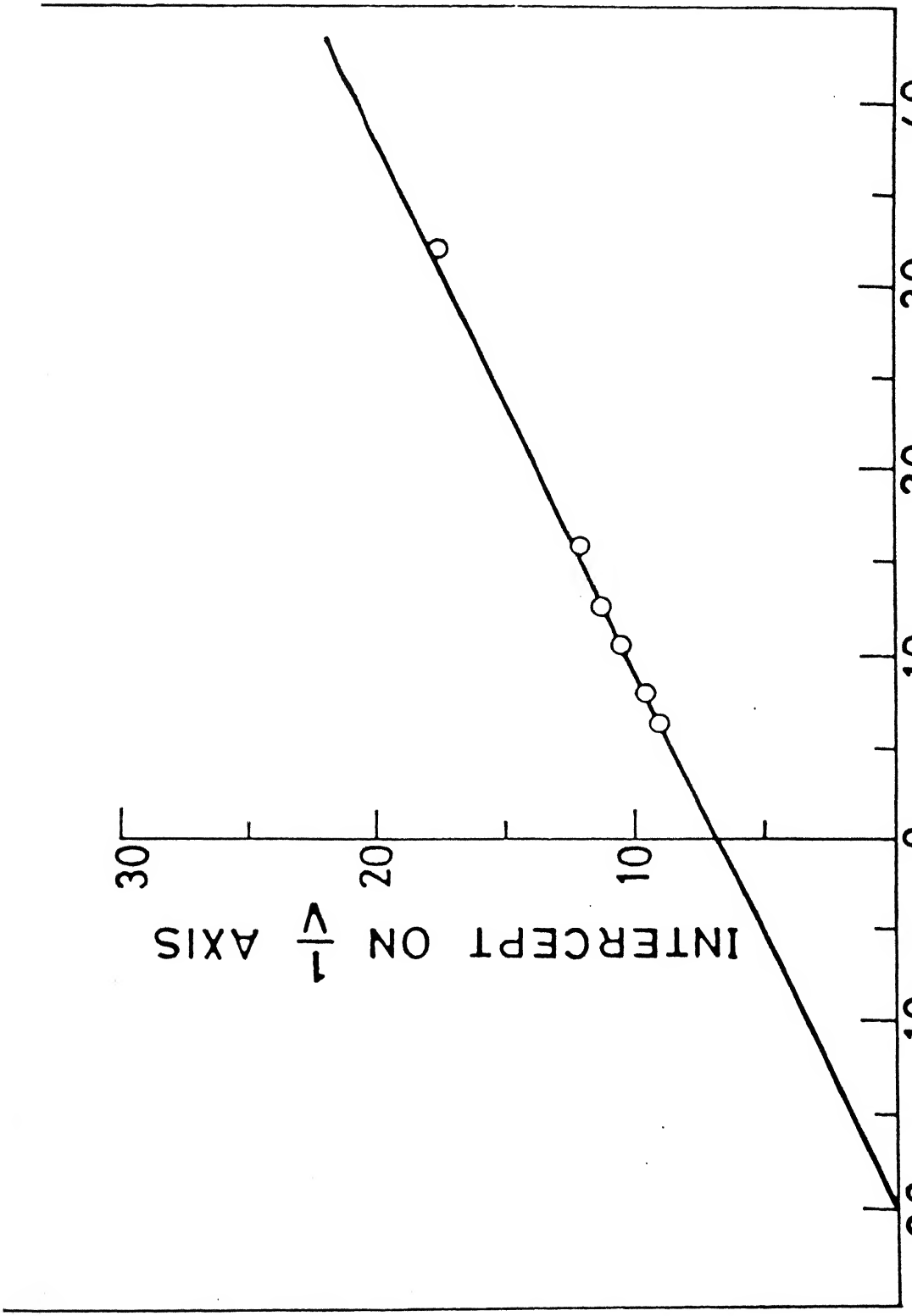


Fig. III.7(A). Secondary plot of intercept on 1/V axis of Fig. 6 vs. $[GSSG]^{-1}$.

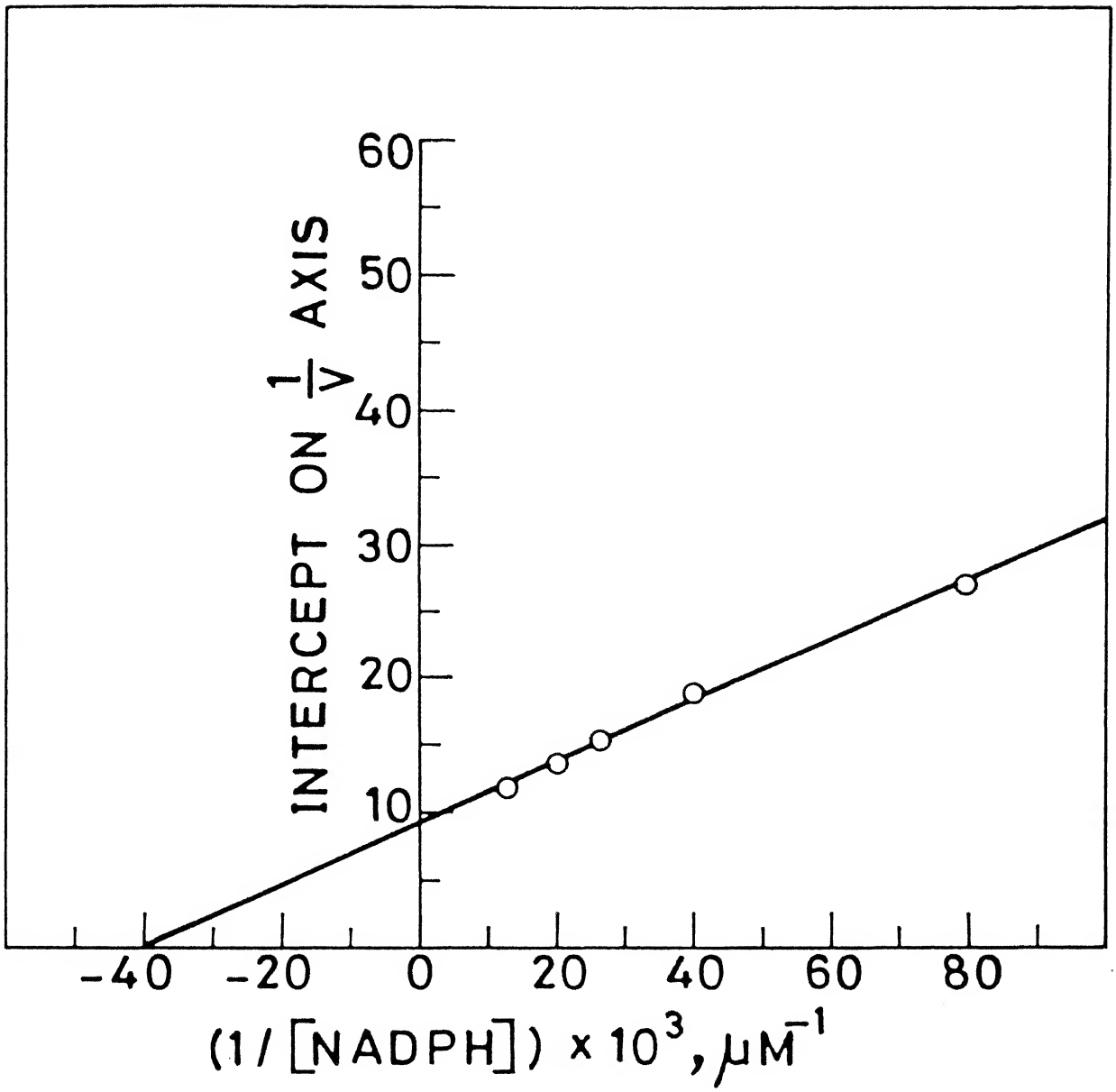


Fig. III.7(B). Secondary plot of intercept on 1/V axis of Fig. 6 vs. $[NADPH]^{-1}$.

and then slowly levels off. The nature of curves are similar to those found in the aqueous system. The shape of these plots demonstrates that the enzyme follows the saturation kinetics at 250 μM GSSG and 100 μM NADPH in reverse micellar solution.

III.3.1.4.2 Determination of Kinetic Parameters of Glutathione Reductase

Fig. III.6 shows the initial velocity pattern for the glutathione reductase as a function of $[GSSG]^{-1}$ at different fixed concentrations of NADPH (12.5 μM, 25 μM, 37.5 μM, 50 μM and 75 μM) in 0.1M CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) at W_o = 29.7 and pH 8.0. The enzyme in reverse micellar solution shows the same initial velocity pattern as obtained in aqueous medium. The nature of double reciprocal plots suggests that glutathione reductase from yeast follows the sequential mechanism in both the aqueous [16] and reverse micellar media. These studies demonstrate that the enzyme in CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) micellar system, follows the Michaelis-Menten Kinetics within a specified concentration range.

Figs. III.7A and 7B show the secondary plots derived from the initial velocity pattern of Fig. III.6. The K_M values for GSSG and NADPH determined from the intercept at the abscissa of Figs. III.7A and 7B are given in Table III.1. The K_M values for these two substrates, GSSG and NADPH, determined at W_o values of 14.4, 20.0, 25.5 and 29.7 at pH 8.0 are also summarized in Table III.1. The K_M values for GSSG are in the range 10-60 μM and those of

Table 1: Kinetic parameters for the glutathione reductase in water[a] and the reverse micelle[b], 0.1M CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) at pH 8.0 at 30°.

Waterpool(W_o) at pH 8.0	GSSG K_M(μM)	NADPH K_M(μM)	k_{cat}(TN) (min^{-1})
29.7	55.0(1028.0)	25.0(467.2)	11764
25.5	52.6(1144.1)	15.6(339.7)	10665
20.0	----	16.6(463.0)	11560
14.4	11.5(420.3)	14.2(549.4)	10120
Water,			
pH 7.6	64	5	13600
8.0	110	-	10660

a. The K_M values taken from [17].

b. Determined by Lineweaver-Burk plots in the linear concentration range (overall) of substrate 30 μM to 200 μM for [GSSG] and 10 μM to 75 μM for [NADPH]. $K_{M(wp)}$ is shown by bracket value and $K_{M(ov)}$ is outside the bracket.

ADPH are in the range 5-25 μM. It is of interest to note that he $K_{M,ov}$ values are quite close to the K_M values obtained in the aqueous medium reported by Moroff et al. [17]. Similar type of results have been reported by Steinmann et al. [25] for lysozyme in CTAB/$CHCl_3$-isooctane system in which the $K_{M,ov}$ values for lysozyme are 0.164 and 0.42 μM at W_o = 20.3, pH 5.0 and W_o=24.7, pH 7.0, respectively. These values are close to the K_M 0.27 μM in water.

In reverse micellar system, unlike the aqueous solution, two types of K_M values are reported. First $K_{M,ov}$ is the Michaelis constant of the substrate with respect to the overall volume of the micellar solution. Second, $K_{M,wp}$ is the Michaelis constant expressed with respect to waterpool. These two K_M values are related as follows [20,29]:

$$K_{M,ov} = K_{M,wp} \cdot f \qquad \text{-----} \quad \text{III.3}$$

where factor, $f = F_W + P(1-F_W)$

F_W is the water volume fraction, i.e. the ratio of volume of water to the overall volume of solution. P represents the partition coefficient of the substrate (if the enzyme is assumed to be soluble only in the waterpool).

$$\text{When} \quad P = 1, \; K_{M,ov} = K_{M,wp} \qquad \text{-----} \quad \text{III.4}$$

For substrates that are preferentially soluble in the waterpool i.e. $p < 1$

$$K_{M,ov} < K_{M,wp} \qquad \text{-----} \quad \text{III.5}$$

and when P = 0 i.e. reagent is only soluble in waterpool

$$K_{M,ov} = K_{M,wp} \cdot F_W \qquad \text{-----} \quad \text{III.6}$$

Since K_M is a good measure of the dissociation constant of the enzyme-substrate (ES) complex, therefore it becomes necessary to decide that which K_M is the more relevant.

The $K_{M,wp}$ values for GSSG and NADPH calculated from the above relation are 10 to 40 times higher than their corresponding $K_{M,ov}$ values in the case of the water system. It is apparent that $K_{M,ov}$ (in reverse micelles) and K_M (in water) are numerically very close. Thus present study supports the concept that $K_{M,ov}$ is a valid K_M in reverse micellar solution since the system behaves as a homogeneous solution and therefore substrate concentration is considered for overall volume. Slight increase in $K_{M,ov}$ values signifies that enzyme-substrate complex is little destabilized as compared to that in aqueous buffer.

III.3.1.5 Spectroscopic Study

Electronic absorption and emission spectroscopy have long been used for elucidating the structural information of enzymes/proteins and other biopolymers in aqueous solution. Transition of enzyme and substrates from aqueous medium to reverse micelles containing organic solvents may result in significant alterations in spectral properties of the reaction system.

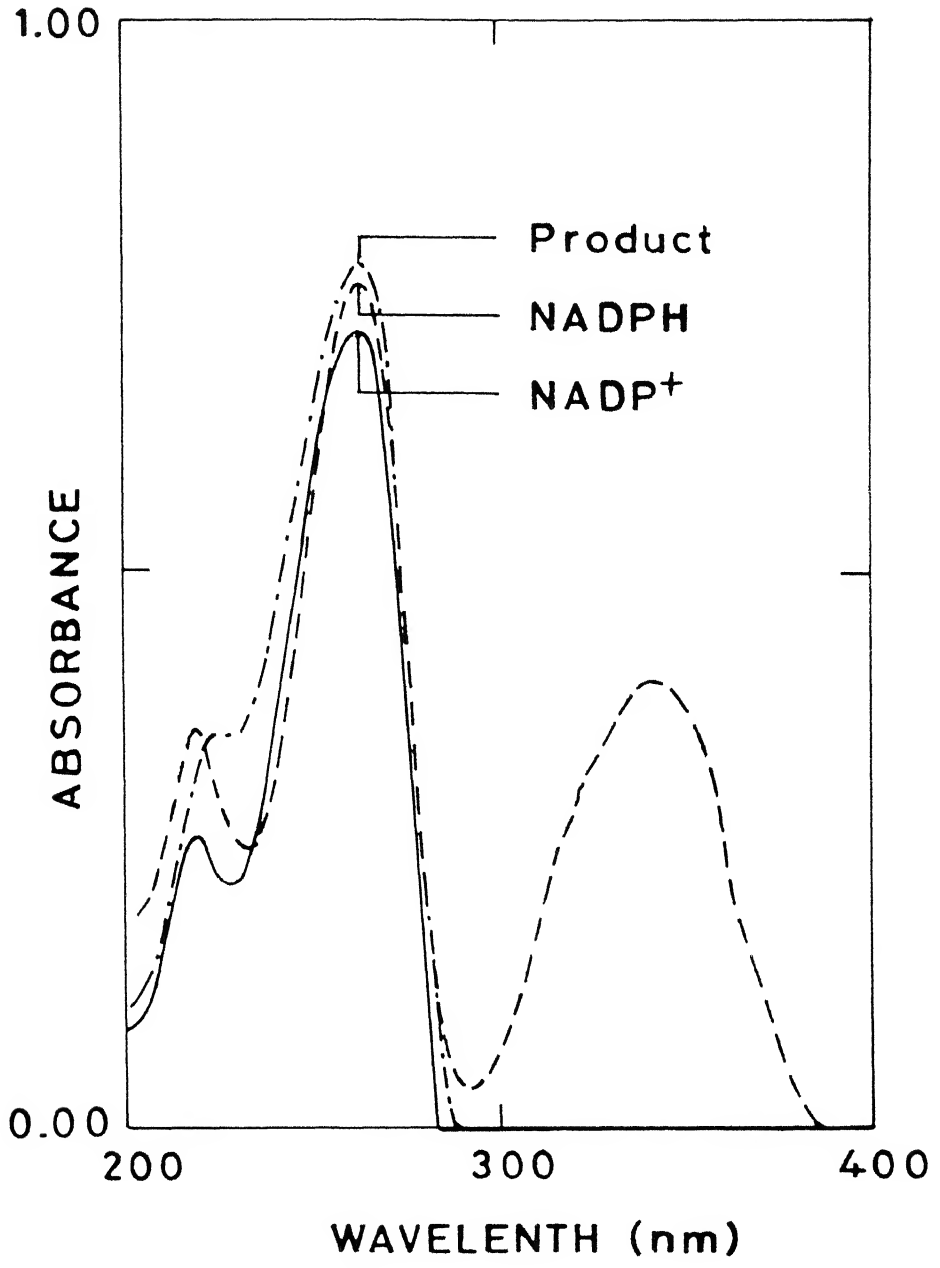


Fig. III.8. Electronic absorption spectra of (——) $NADP^+$; (---) NADPH containing reaction system before start of the reaction; and (-·-·-) product formed after the completion of enzymic reaction in aqueous buffer (0.1 M potassium phosphate , 1 mM EDTA, pH 7.5).

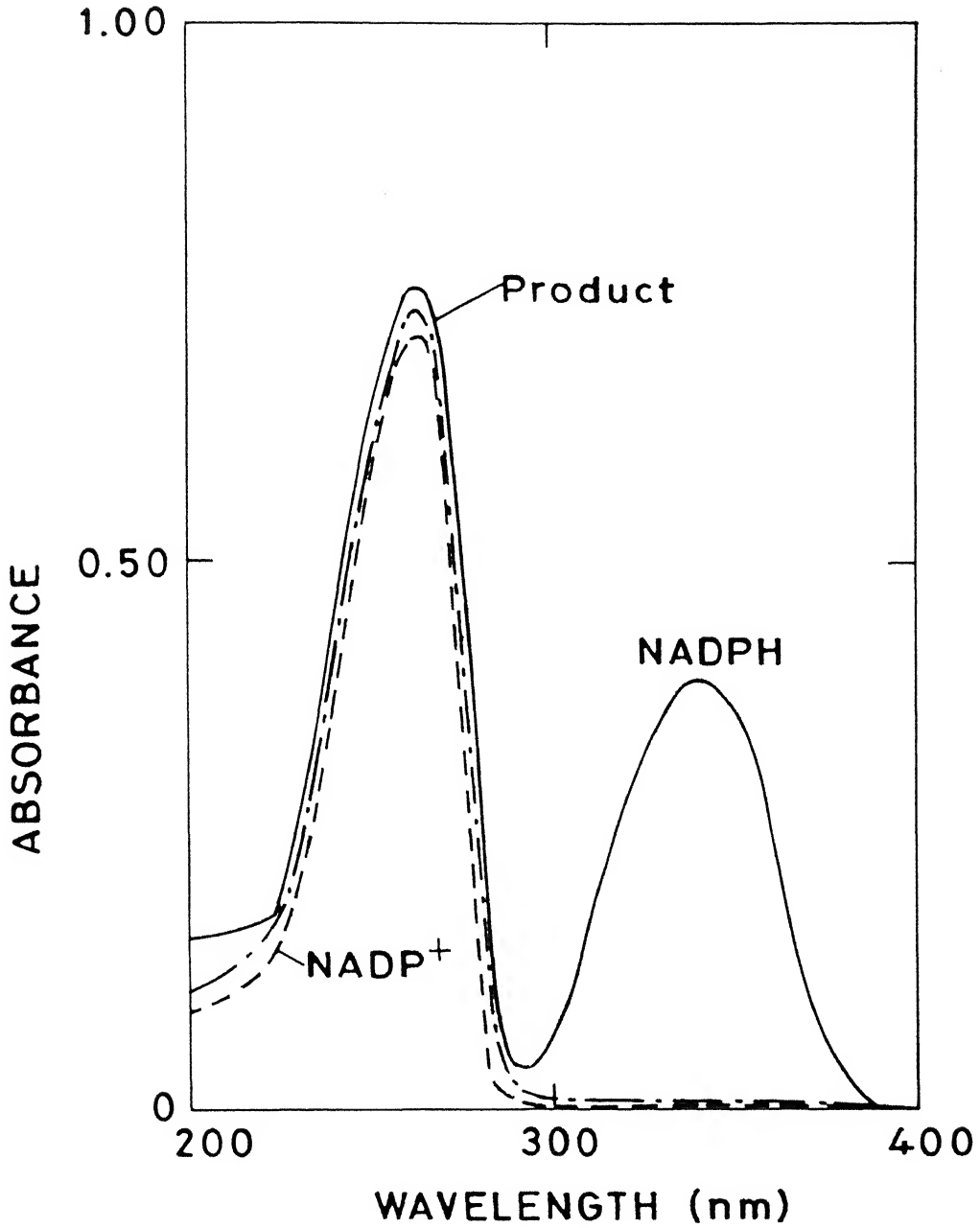


Fig. III.9. Electronic absorbance spectra of (---) $NADP^+$; (——) NADPH containing reaction system ; (·—·—) product formed after the completion of enzyme reaction in reverse micellar solution of 0.1 M CTAB/$CHCl_3$-iso-octane (1:1,v/v) at W_o= 29.7, pH 8.0. Buffer used was same as in the case of Fig. III.8.

In the first instance, to examine the nature of enzyme reaction in water and micellar solution, we studied the u.v.-vis. spectra of the complete reaction system in both the media. Fig. III.8 and Fig. III.9 show the absorption spectra of coenzyme NADPH, $NADP^+$ and the corresponding product (after the completion of enzyme reaction) of glutathione reductase catalyzed reaction, in both aqueous and 0.1 M CTAB/H_2O/$CHCl_3$-isooctane reverse micellar solution respectively. Characteristic absorption peaks of NADPH and $NADP^+$ are obtained at (260 nm, 340 nm) and 260 nm respectively. The nature of the spectrum, is identical in both the aqueous and micellar solution. These data demonstrate that NADPH remains stable in the CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) reverse micellar system and the decrease in absorbance at 340 nm during the assay is solely due to the enzymic oxidation of NADPH to $NADP^+$ in presence of GSSG.

In order to answer the question whether the micellar environment induces structural and, in particular, conformational transitions of glutathione reductase, u.v. absorption and fluorescence spectra have been recorded. The spectra of glutathione reductase in reverse micellar solutions showed no significant shift in the λ_{max} in comparison to water.

III.3.1.6 Time Dependent Stability

One of the fundamental problems for the adoption of micellar enzymology in biotechnology is time-dependent stability of enzymes in this unique media. Fig. III.10 shows the stability of

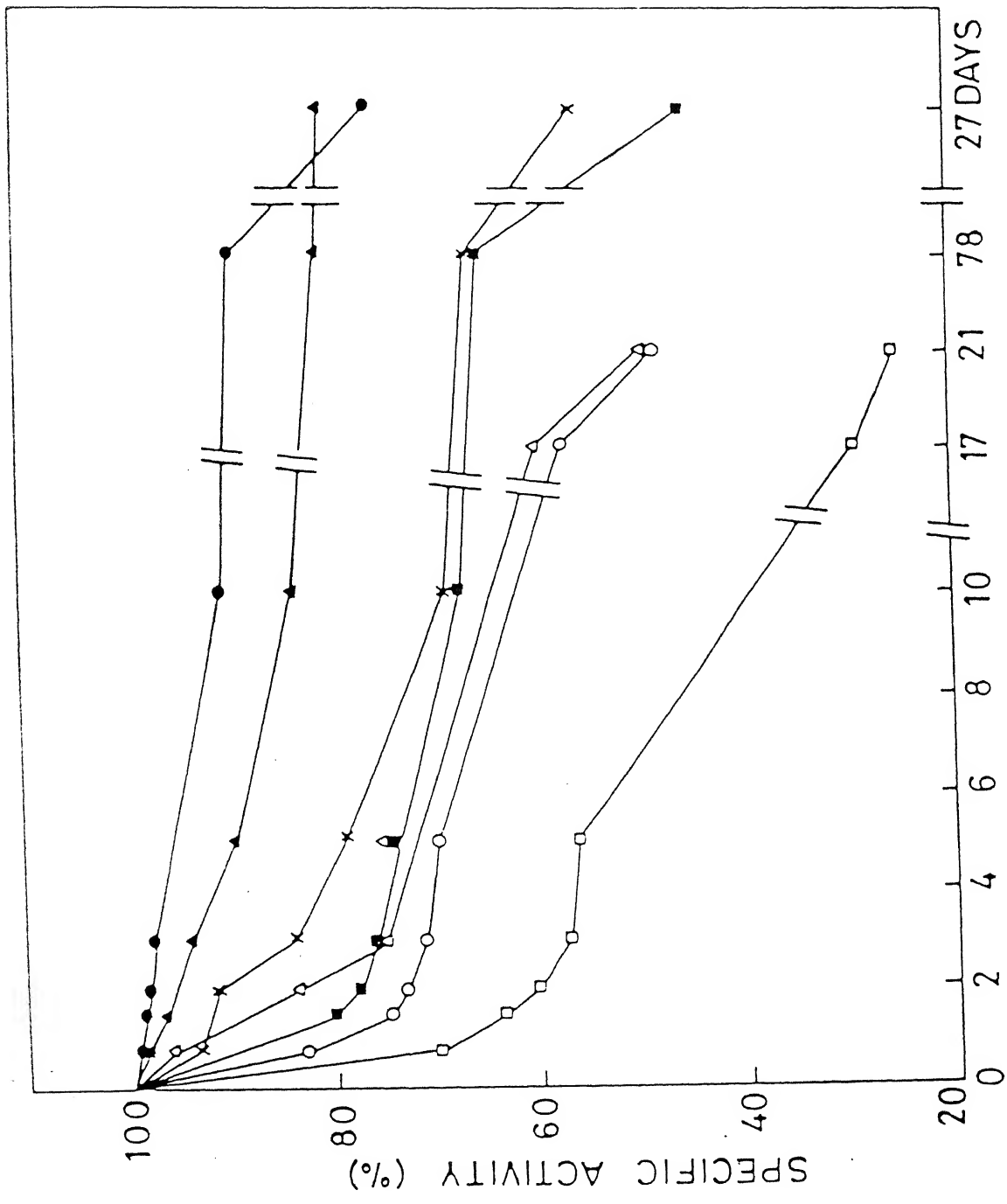


Fig. III.10. Time dependent stability of glutathione reductase in water and 0.1 M CTAB/H_2O/$CHCl_3$-isooctane(1:1,v/v) at different W_o: (-□-□-), W_o= 14.4; (-O-O-), W_o= 29.7; (Δ-Δ-) water, pH 7.5 [in absence of GSSG].(-■-■-) W_o 14.4; (-x-x-) W_o= 20.0; (-▲-▲-) W_o= 29.7; (-●-●-) water, pH 7.5 [in presence of GSSG]. [E]= 0.16 μg/ml. [GSSG] = 250 μM.

glutathione reductase in terms of percent residual activity in the presence and absence of the substrate GSSG in 0.1M CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) reverse micelles as a function of incubation time at different W_o at fixed pH 8.0. Control was determined by measuring the activity of enzyme at zero time. A parallel and similar experiment was run for the observation of time-dependent stability in aqueous medium. The residual activity of glutathione reductase in reverse micellar solution remained approx. 80% at W_o = 29.7 and pH 8.0 even up to about a month, whereas it drops down to 55 and 45% at W_o values of 20.0 and 14.4 respectively. The efficiency of waterpool size (W_o) for the retention of time-dependent stability in the CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) was in the order of 29.7 >> 20.0 > 14.4. The substrate GSSG helped to protect the enzyme stability, probably by masking the active site of glutathione reductase against direct exposure to the organic solvent. The residual activity of the enzyme found in the micellar solution at the higher W_o value of 29.7 and pH 8.0 was almost identical to that in the aqueous medium. Thus CTAB/H_2O/$CHCl_3$-isooctane (1:1, v/v) medium maintains the activity and time dependent stability of glutathione reductase almost to the same extent as aqueous medium under certain conditions. Only a few enzymes such as peroxidase [26], α-chymotrypsin [21], hydrogenase [30], etc. have been reported to maintain better stability in reverse micellar media than that in aqueous system. After 24 hours at pH 8.5, 25% of the original activity of trypsin is lost in buffer and 70% in reverse micelles [31]. Lee and Biellmann [5] found cholesterol

oxidase to be stable for 24 h. Han and Rhee [24] showed that lipase lost 12% activity in 12 h. Robinson and coworkers [9] reported that α-chymotrypsin lost 30% activity in 2.5 h. In the case of dihydrofolate reductase [32] Katiyar et al. observed that after 48 hr. in aqueous solution, the enzyme loses its activity by about 90%, whereas, only about 50% activity is lost in CTAB reverse micelles in $CHCl_3$/isooctane at W_o = 7.20, pH 7.0. The activity of malate dehydrogenase [33] on incubation in CTAB reverse micelles for 3 hr drops down to almost zero, whereas in water, it takes 24 h to fall down to zero.

III.3.2 Study on the Glutathione Reductase from Bovine Intestinal Mucosa

Enzymes with the same name but obtained from different organism often have different amino acid sequences and hence different properties and catalytic activities. It is also known that enzyme from different sources which catalyzes a given reaction will not always have the same molecular structure or necessarily the same kinetics. Keeping this in mind, the behavior of same enzyme from different sources in microcaptive environment of reverse micelles in non-polar solvents, has been investigated.

The enzyme from bovine intestinal mucosa (BIM) was solubilized in the similar way as described in the case of yeast enzyme. The activity of this enzyme has been investigated while entrapped in a series of cationic reverse micelles prepared by tetradecyl-

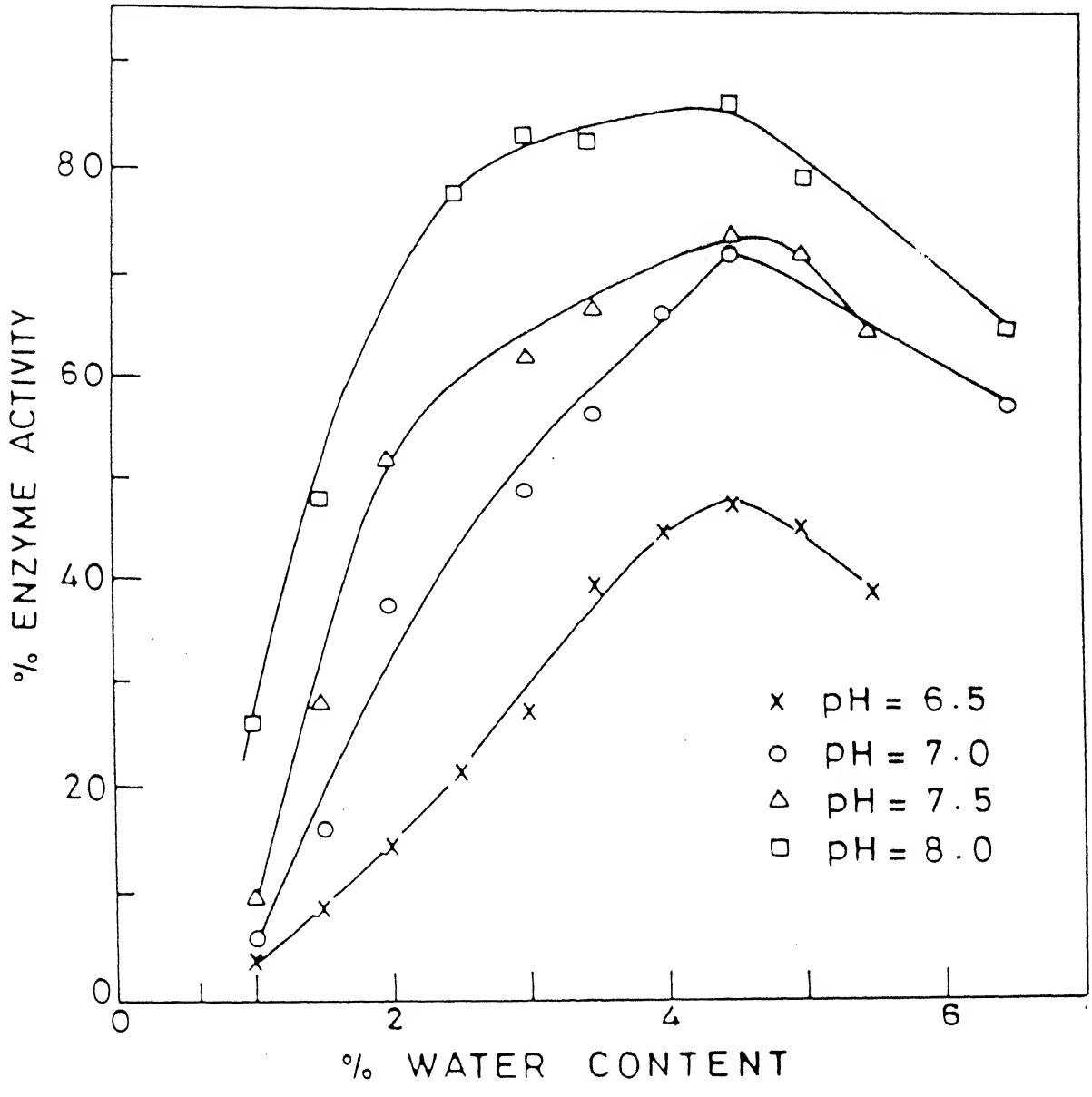
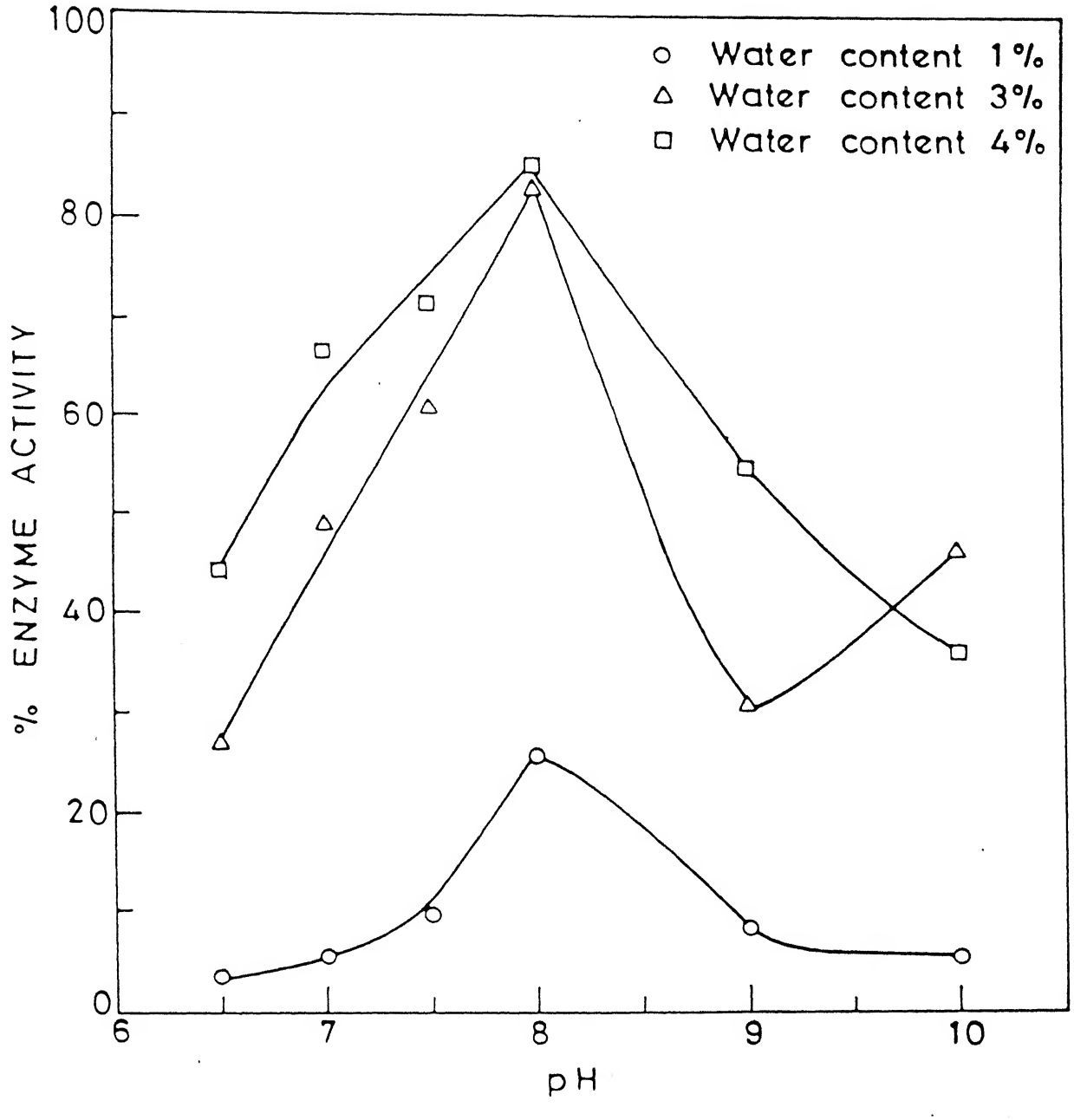


Fig. III.11. Effect of water content on the activity (in terms of percent of actvity in water) of B.I.M. glutathione reductase in the reverse micelles of 4% cetrimide in $CHCl_3$-isooctane (1:1,v/v) at different pH: (x) pH 6.5; (O) pH 7.0; (Δ) pH 7.5; (□) pH 8.0. Concentrations are: [GSSG] = 250 μM; [NADPH] = 100 μM; [E] = 0.2 μg/ml. Buffer used is 50 mM potassium phosphate.

trimethylammonium bromide (TDTAB), cetyltrimethylammonium bromide (CTAB) and mixed alkyltrimethylammonium bromide (cetrimide) in presence of the solvent system $CHCl_3$-isooctane respectively. It was found that the enzyme from B.I.M. maintained its catalytic efficiency in these surfactants containing non-polar solvents. The catalytic efficiency of the enzyme varies for all different surfactants. However, the variation is not very significant. In all these cases, the rate of the reaction catalyzed by B.I.M. glutathione reductase was regulated by the variation of different parameters like pH, water content in the waterpool, enzyme and substrate concentration etc.

III.3.2.1 Effect of Water Content

Similar to yeast glutathione reductase, the enzyme from B.I.M. showed marked dependence on the water content entrapped into the reverse micelles. Fig. III.11 shows the variation of enzyme activity as a function of amount of water (expressed as % water content) inside the 4% cetrimide/$CHCl_3$-isooctane (1:1, v/v) at different pH. At each pH value the water content profile shows a bell shaped curve. The percent of enzyme activity in this system has been calculated with respect to the specific activity of B.I.M. glutathione reductase in aqueous system, i.e. 0.1 M potassium phosphate, 1 mM EDTA, at pH 7.5. From the figure it is evident that the activity of the enzyme is low at low water content, showing an increase with increase in water content and achieving a maximal value at 4.5% water content at different pH.



ig. III.12. Variation of percent of B.I.M. glutathione reductase activity with the function of pH at different water content: (0) 1.0%, (Δ) 3.0% and (□) 4% in reverse micelles of 4% cetrimide in $CHCl_3$-isooctane(1:1,v/v). Concentrations are same as in Fig. III.11.

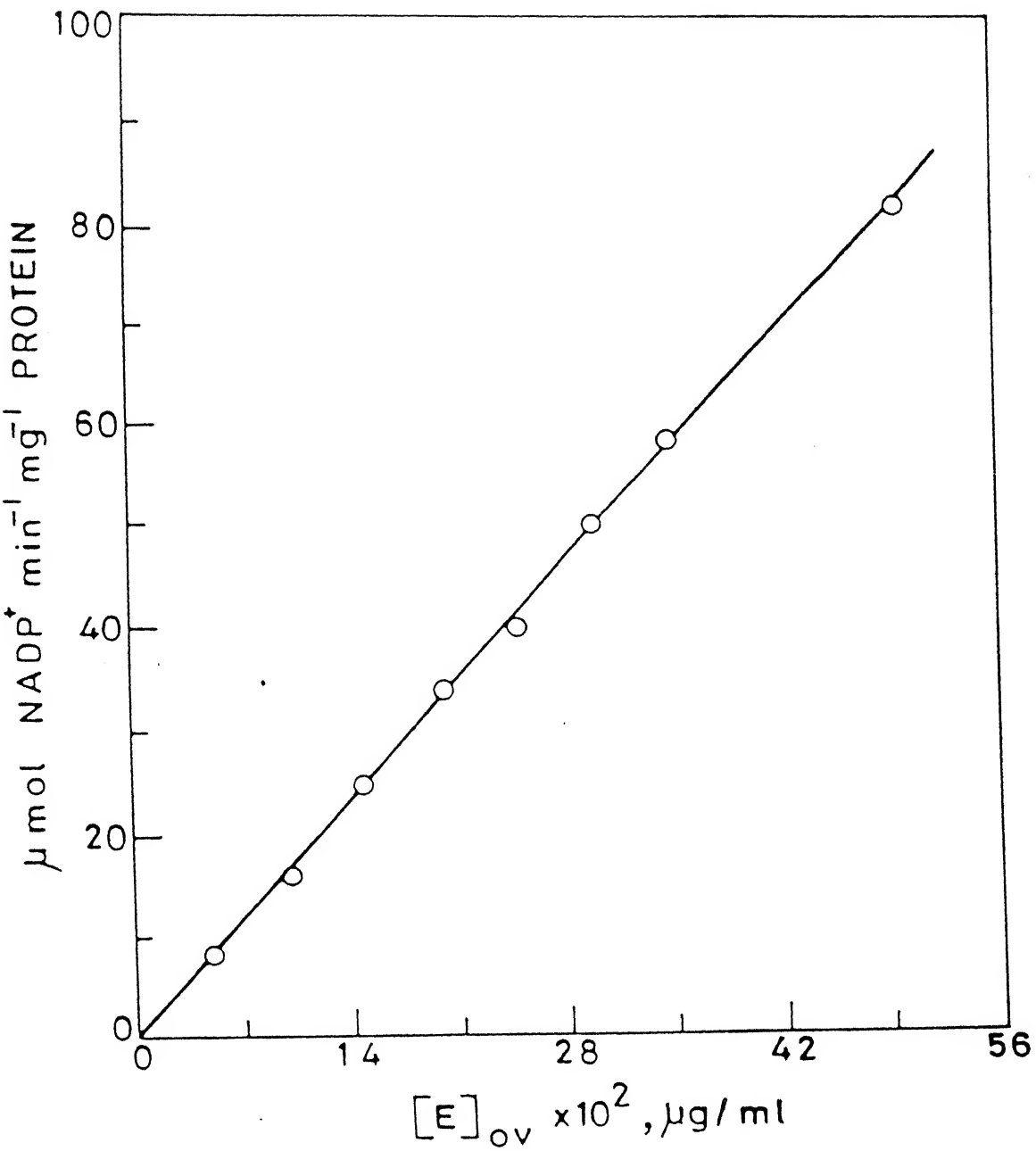
Thus optimum water content for the maximum activity of enzyme at pH's 6.5, 7.0, 7.5 and 8.0, is 4.5% in the reverse micelles of 4% cetrimide in $CHCl_3$/isooctane (1:1, v/v).

III.3.2.2 Effect of pH on the Activity of Enzyme

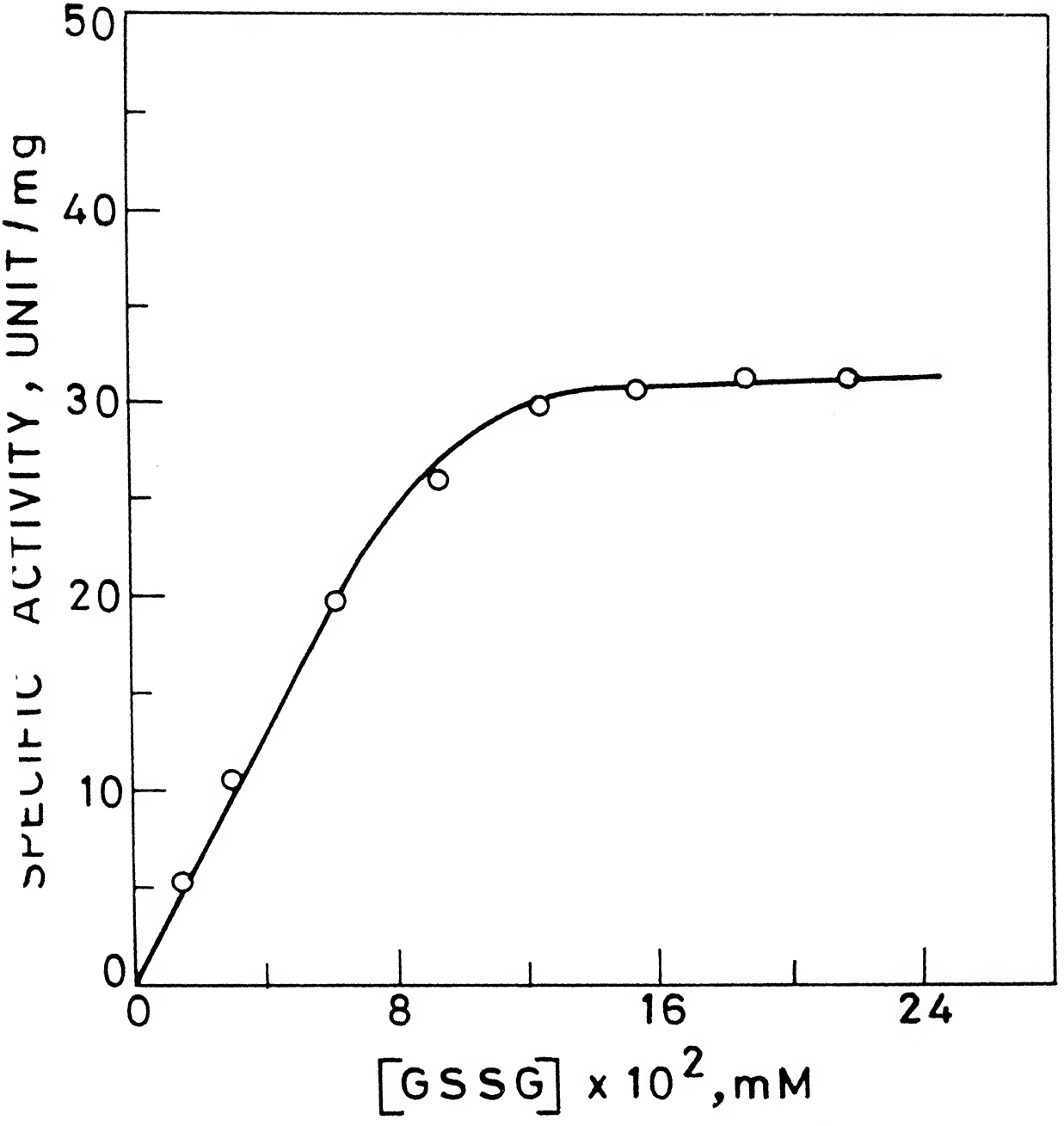
Fig. III.12 represents the dependence of enzyme activity on pH of the buffer transferred inside the reverse micelles at different amount of water content in 4% cetrimide/H_2O/$CHCl_3$-isooctane system. In each case, the enzyme shows a bell-shaped curve. The maximum activity of B.I.M. glutathione reductase has been observed at pH 8.0. The highest activity of the enzyme in cetrimide/H_2O/$CHCl_3$-isooctane reverse micellar solution was about 85% to that obtained in the aqueous solution at optimum pH. Very small shift by increase of 0.5 unit in the optimum pH of enzyme in non-polar medium, has been found in comparison to that in water. It may be noted that the similar effect was observed in the case of yeast glutathione reductase. These observations show that the enzyme from both sources exhibits identical nature of waterpool and pH profile in the cationic reverse micelles.

III.3.2.3 Effect of Enzyme Concentration

The effect of enzyme concentration on the initial rate of reaction provides significant information about purity of the enzyme. Normally, there is linear relationship between the velocity and the enzyme concentration. Frequent departures from linearity are due to an artefact of the assay system, but in rare cases, such behavior is due to the property of enzyme itself



III.13. Variation of initial rate of B.I.M. glutathione reductase catalyzed reaction with concentration of enzyme in 4% cetrimide/$CHCl_3$-isooctane (1:1,v/v) at 4% water content, pH 7.0. Concentrations are [GSSG] = 250 μM; [NADPH] = 100 μM; [E] = (0.05 - 0.6 μg/ml.



ig. III.14(A). Dependence of B.I.M. glutathione reductase activity on the GSSG concentrations at fixed [NADPH]= 100 μM, in reverse micelles of 4% cetrimide in $CHCl_3$-iso-octane (1:1,v/v) solution at 4% water content, pH 8.

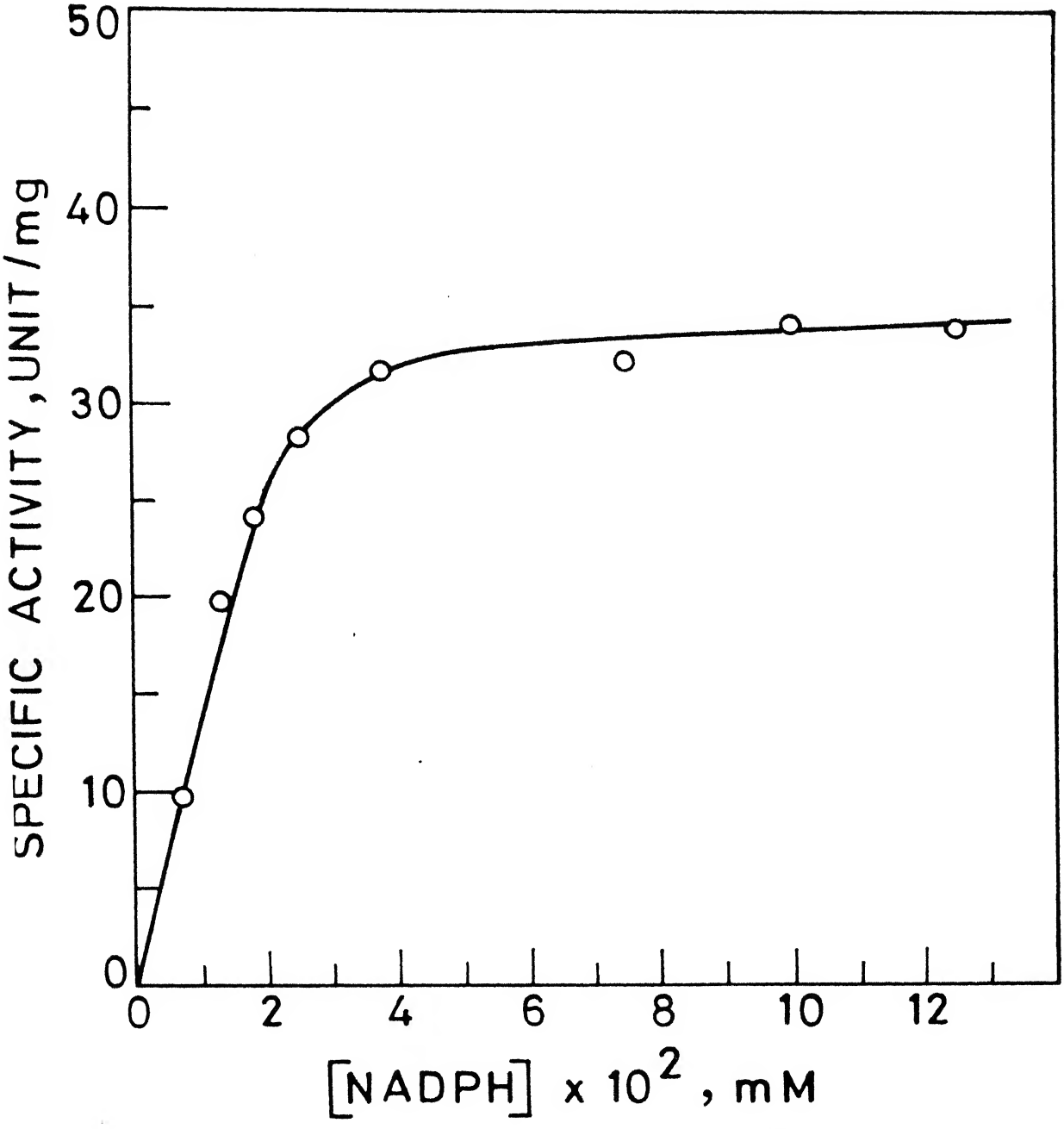


Fig. III.14(B). Dependence of B.I.M. glutathione reductase activity on the NADPH concentrations at fixed GSSG in reverse micelles of 4% cetrimide in $CHCl_3$-isooctane (1:1, v/v) solution at 4% water content, pH 8. [E] = 0.2 μg/ml.

[34]. In reverse micelles enzyme molecules are made to reside and function in the waterpool (i.e. tiny water droplets) of reverse micelles. It is necessary to know whether the enzyme molecules behave separately in this captive microenvironment. Fig. III.13 shows the rate data for enzyme catalyzed reaction at fixed substrate concentrations as a function of varying enzyme concentrations. The data show that the rate of reaction is proportional to the total amount of enzyme present. It may be noted that the reaction velocity increases linearly up to 0.6 μg/ml of enzyme in reverse micelles of 4% cetrimide in $CHCl_3$/ isooctane (1:1, v/v), indicating thereby that the enzyme molecules of glutathione reductase from B.I.M. function independently in the reverse micellar solution. This result also shows the homogeneity of enzyme solution in this new media.

III.3.2.4 Effect of Substrate Concentration on the Enzyme Reaction Velocity

Substrate concentration is one of the important factors which determines the velocity of enzyme reactions. Since, the activity of enzymes from different sources is often different, it may be affected differently by the change in concentration of substrates. Figs.III.14 A and 14 B show the dependence of initial rate of enzyme reaction (expressed in terms of specific activity) with the variation of concentration of oxidized glutathione [GSSG] at fixed [NADPH] and vice versa in cetrimide/$CHCl_3$-isooctane system at pH 8.0 and 3% water content. As with yeast glutathione reductase, the hyperbolic form of typical substrate

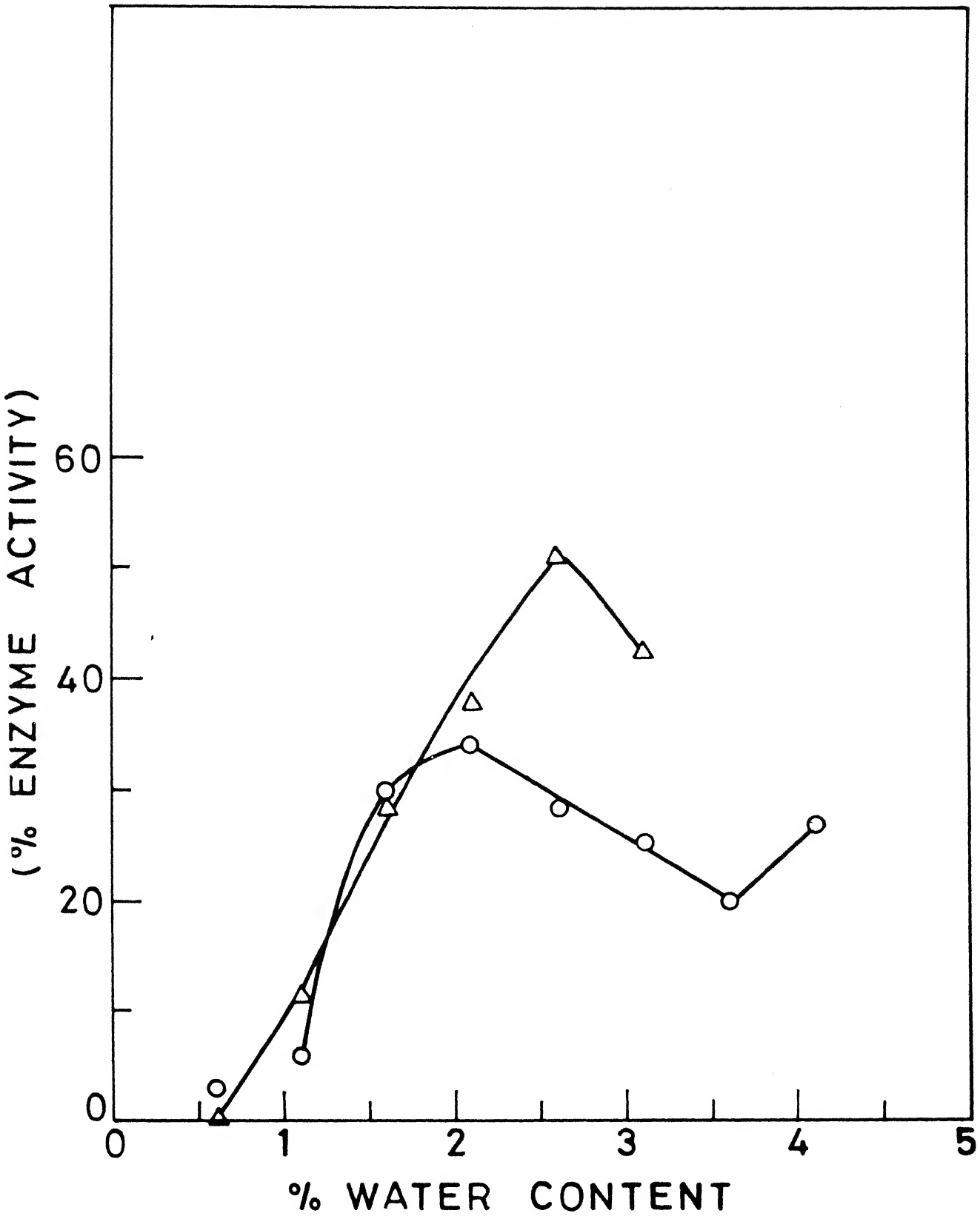


Fig. III.15 Variation of percent of B.I.M. glutathione reductase activity as a function of water content at pH 7.5 in 0.1 M concentration of (△) CTAB and (○) TDTAB : CTAB (3:1) in $CHCl_3$ -isooctane (1:1,v/v). Concentrations are same as in other cases.

concentration curve obtained in the reverse micelles, suggests that glutathione reductase from B.I.M. source obeys Michaelis-Menten Kinetics within the specified concentration range in the heterogeneous medium.

III.3.2.5 Effect of Different Surfactants

The rate of enzyme reaction in reverse micellar solution is highly dependent on the choice of different surfactants. The shape, size and structure of reverse micelles is governed largely by the type of surfactant and solvents, which ultimately lead to marked change in the properties of waterpool inside the reverse micelles. The study on the effect of surfactant provides the information for optimization of enzyme activity in reverse micelles.

Fig. III.15 depicts the variation of enzyme activity of B.I.M. glutathione reductase as a function of water content of reverse micelles generated separately from two surfactants viz. cetyltrimethylammonium bromide (CTAB) and the mixture of tetradecyltrimethylammonium bromide (TDTAB):cetyltrimethylammonium bromide (3:1) respectively. In both surfactant systems, the plots are bell-shaped. The enzyme shows maximum activity at 2.5% water content and 1.5% water content in the reverse micelles of CTAB and the surfactant mixture of TDTAB & CTAB respectively. Under this experimental condition, the maximum activity of the enzyme was about 60% in CTAB and ~35% in the surfactants mixture. These results indicate that cationic reverse micelles provide a

suitable environment for the study of B.I.M. glutathione reductase in the non-polar solvents.

In the mechanism of catalysis by glutathione reductase, reduction of disulfide leads to formation of a transient adduct (probably a charge transfer complex) between cys-46 and C4α of FAD. In the next step of catalytic cycle, the mercaptide ion of cys-41 attacks the S-S bond of the bound GSSG to form a mixed protein-glutathione disulfide and to release GSH [35]. This nucleophilic attack may be facilitated by a neighbouring histidine or lys-49. In a non-polar solvent, the Vander Waal and hydrogen bonding interactions in the protein glutathione disulfide charge transfer complex might be disturbed due to the perturbations of active site residues by the change in dielectric constant and microproperties of milieu. The reverse micellar waterpool probably provides a favorable medium for the above mechanistic process. There is a possibility of designing better conditions by adjusting different parameters such as W_o, pH, solvent component ratio, temperature ionic strength etc. whereby the enzyme can show still more enhanced activity and stability with respect to aqueous system.

III.4 Conclusion

The enzyme glutathione reductase in cationic reverse micelles in $CHCl_3$/isooctane (1:1, v/v) solvent mixtures exhibits an activity that is almost comparable to that in aqueous system. The time-dependent stability of yeast glutathione reductase in

reverse micelles is the same as that in water at W_o = 29.7 and less at W_o < 29.7 at pH 8.0. The kinetic characteristics do not show significant changes. Initial velocity patterns indicate that the enzyme mechanism does not undergo any change in transfer from aqueous solution to reverse micellar medium. The $K_{M,ov}$ value in reverse micelles is numerically close to the value of K_M in water.

Our results show the similarities between the enzyme glutathione reductase from two sources. Both the enzymes from yeast and B.I.M. in cationic reverse micelles exhibit the catalytic activity comparable to those in aqueous solution. They follow the Michaelis-Menten Kinetics within the specified experimental conditions. In general, our data emphasize the role of water and pH of the waterpool in modifying the enzyme's behavior in reverse micelles.

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CHAPTER IV

CATALYTIC EFFICIENCY AND KINETIC PROPERTIES OF A COUPLED ENZYME SYSTEM (PYRUVATE KINASE + LACTATE DEHYDROGENASE) IN THE WATER RESTRICTED ENVIRONMENT OF NON-AQUEOUS SOLVENTS

IV.1 Introduction

In vitro studies of enzymes are usually conducted in aqueous solution. However, in vivo most of the enzymes function at or near the membrane of the living cell. Few enzymes are embedded or buried in the interior of the membrane. The notion that all proteins should be studied in aqueous media is not derived from the inherent properties of proteins, but rather from technical limitations which have largely confined the study of proteins to those which are water soluble [1]. The proteins which are located within the non-aqueous hydrocarbon core of the membrane could be expected to retain their native conformations in non-aqueous solvents. It is difficult to see how this dual environment may be mimicked in a bulk solution. Naturally, the traditional enzyme assay may not reveal the real catalytic or biological reality of enzymes inside the living system. This fact is also clear from the phenomena that many enzymes/proteins after removal from cellular membrane to the ordinary aqueous

system, stop functioning [2-3]. These proteins require proper media which should mimic the membrane like environment.

To simulate *in vitro* the conditions of enzyme action *in vivo*, various attempts have been made by many workers but no model has been found to be adequate. Protein engineering for the modification of enzymes is in very embryonic stage. Modification of amino acids either by changing with others or by attaching some non-polar groups with the amino groups, is very specific for a couple of cases only [4-6]. Enzymatic studies in mixtures of water and organic solvents with a high concentration of non-aqueous components, are also far from ideal. Interestingly, reverse micelles which are the aggregates of surfactants (amphiphilic molecules) in non-aqueous solvents, provide a microenvironment inside their core [7]. This is also established that the water inside the waterpool of reverse micelles has unique properties. Various groups like Luisi et al. [8-10], Martinek et al. [11-13], Fletcher et al. [14] and Laane et al. [15,16] and Katiyar et al. [17,18] have demonstrated that reverse micellar media provide the suitable environment for the study of many enzymes in vitro. They have studied several enzymes like α-chymotrypsin, trypsin, lysozyme, ribonuclease, peroxidase, alcohol dehydrogenase, malate dehydrogenase, dihydrofolate reductase etc. in the water-restricted microcaptive environment in non-polar solvents. Most of them show the comparable activity to that in aqueous media. However, very few enzymes have been reported to exhibit superactivity (i.e. the activity in reverse

micellar media is greater than that in aqueous media) [9,11,19,20]. Several enzymes are not functional too under ordinary conditions. In the third chapter, an exhaustive investigation on the kinetic characteristics of glutathione reductase (from yeast and bovine intestinal mucosa) have been reported. The maintenance of significant stability and activity by single enzyme system (glutathione reductase) in reverse micellar media, in turn, prompted us to investigate the kinetic behavior of a combined enzyme system. Study on the subunit-subunit and protein-protein interactions within the restricted aqueous environment may provide very interesting results on the understanding of catalytic and structural behavior of enzymes that function together in a series of transformations. In order to understand the behavior of multi and complex enzyme systems detailed studies on different class of combined enzymes are required.

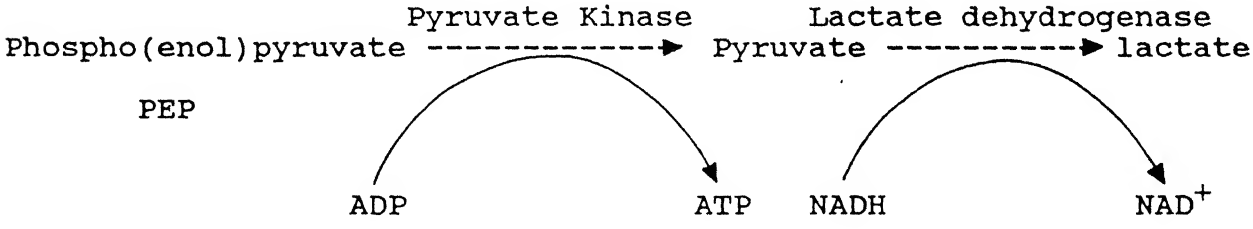
Pyruvate Kinase catalyzed reaction is an interesting system which has been chosen for study. The enzyme pyruvate kinase is a cytoplasmic enzyme but is reversibly adsorbed on membranes. It is a key enzyme in glycogen metabolism. It has found extensive use in the quantitative determination of ADP and that of enzymes that catalyze the formation of ADP. The enzyme from rabbit muscle is tetrameric (subunit M_r 57,000 dalton) having overall molecular weight 2,40,000 dalton with four metal binding sites. In the form of the polyenzyme system the enzyme catalyzes the concerted reaction followed by lactate dehydrogenase.

Lactate dehydrogenase (LDH) is also a complex enzyme having four subunits and M_r 1,45,000 dalton. In anaerobic glycolysis, LDH is the terminative enzyme in the sequence of reactions that promote the breakdown of glucose to lactate, therefore it is essential for the production of ATP, an efficient energy carrying system in cells. Lactate dehydrogenase catalyzes the following reaction [21].

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightleftharpoons[\text{dehydrogenase}]{\text{lactate}} \text{lactate} + NAD^+$$

Scheme IV.1

The scheme of the catalysis by the coupled enzymes is shown below [22]:



Scheme IV.2

Todate, only a couple of combined enzyme systems like hydrogenase [23], cholesterol oxidase [24] etc. have been solubilized and studied in different reverse micellar systems.

This chapter presents an investigation on the activity of the double enzyme system (namely pyruvate kinase and lactate dehydrogenase) in the reverse micelles of cationic surfactant

cetrimide in $CHCl_3$-isooctane (1:1, v/v). The study on this double enzyme system in cetrimide/$CHCl_3$-isooctane (1:1, v/v) reverse micellar solution would reveal the behavior of enzymes in this water restricted captive environment.

IV.2 Experimental Section

IV.2.1 Materials

Enzymes pyruvate kinase and lactate dehydrogenase both from rabbit muscles were obtained from Boehringer-Mannheim GmbH, W. Germany. Phospho(enol)pyruvate (PEP), (trisodium salt, 97% purity), Adenosine 5'-diphosphate (ADP) from equine liver (grade IV, 95% purity) and β-Nicotinamide adenine dinucleotide, reduced form (NADH) from yeast, grade III were purchased from Sigma Chemical Co., St. Louis, U.S.A.

The surfactant cetrimide (mixed alkyltrimethylammonium bromide) was a product from Sigma Chemical Co. U.S.A. The organic solvents isooctane, puriss grade and chloroform, HPLC and spectroscopic grade were procured from Fluka, Switzerland and s.d. fine chemicals Ltd. Bombay, India respectively.

All the buffer components triethanolamine hydrochloride, potassium phosphates, glycine and trizma base were purchased from Sigma Chemical Co. Other chemicals potassium chloride, $MgSO_4.7H_2O$, potassium hydroxide, hydrochloric acid (all AR quality) were from Merck, India. Throughout the experiment, all quartz-double distilled water was used.

IV.2.2 Methods

IV.2.2.1 Purity of Enzymes

Enzymes were dialyzed against 0.1M potassium phosphate buffer solution. The homogeneity of the preparations was checked by established methods such as SDS polyacrylamide gel electrophoresis and gel permeation chromatography as described in the previous chapter [25].

IV.2.2.2 Enzymes and Substrates Containing Reverse Micelles

Reverse micellar solutions containing desired concentration of substrates were generated by injecting, by microsyringe, required volumes (2-4 μl) of aqueous stock solution of each of phospho(enol)pyruvate (60 mM), ADP (100 mM) and NADH (50 mM) into the solution of 4% cetrimide in $CHCl_3$-isooctane (1:1, v/v) such that the overall volume of the assay mixture was 1.0 ml. The aqueous stock solution (2-10 μl) of the enzymes (pyruvate kinase, 0.1 mg/ml and lactate dehydrogenase, 0.05 mg/ml) were solubilized into the micellar solution by the same technique as described in chapter III. The water content in the waterpool of reverse micellar solution was adjusted by injecting additional amount of 250 mM $KCl/MgSO_4$ (1:1) containing 50 mM triethanolamine-KOH or potassium phosphate buffer at different pH (5.8-10.0). The pH of the solution reported in the investigation is the pH of the aqueous solution transferred to the micellar solution. The reaction mixture was agitated using a vortex mixture for a few seconds for solubilization of aqueous solution into reverse micelles.

IV.2.2.3 Enzyme Activity Measurement

In practice, for assay of coupled enzymes, the basic principle is that a steady state should be set up in which the rate of action of the indicator enzyme is equal to that of the measured enzyme. The enzyme activity was measured with a Gilford-260 uv/visible spectrophotometer by recording the decrease in absorbance at 340 nm with time. All the experiments were conducted at 30 ± 0.1°C. The reaction was started by the addition of enzyme mixture (required for maintaining the steady state of the reaction) to the rest of the sample solution containing substrates. The negligible auto oxidation-reduction of substrates, in the absence of enzyme was checked before the final measurement of rate of reaction. The specific activity of pyruvate kinase determined by the method reported earlier [26]. The same technique was followed for the assay of pyruvate kinase and lactate dehydrogenase in reverse micellar system where only reverse micellar solution without reagents was kept in reference cell. The details of the assay procedures for lactate dehydrogenase and pyruvate kinase are given below:

Assay of Lactate Dehydrogenase

The activity of lactate dehydrogenase was measured in 0.1M potassium phosphate buffer, pH 7.0 by recording the decrease in absorbance at 340 nm with time. The overall concentration of the substrates and the enzyme were as follows:

[NADH] = 0.2 mM, [Sodium pyruvate] = 1 mM and $[LDH]_{ov}$ = 0.1 μg/ml

The specific activity of lactate dehydrogenase determined in aqueous solution was 460 units/mg. One enzyme unit oxidizes one μmole of NADH per minute at 30°C, pH 7.0.

Assay of Pyruvate Kinase

Activity of pyruvate kinase was measured continuously by coupling the pyruvate formation to the lactate dehydrogenase reaction (NADH utilization). The change in optical density at 340 nm per unit time is a measure of pyruvate kinase activity at the steady state condition of reaction system. The concentration of the stock solution of reagents and enzymes used in both the media are:

(i) 0.1M TEA-KOH or 0.1M potassium phosphate buffer, pH 7.5

(ii) 0.25M $MgSO_4.7H_2O$/KCl solution (1:1) in water

(iii) 60 mM phospho(enol)pyruvate in 0.25M salt solution

(iv) 100 mM ADP in 0.25M salt solution

(v) 0.05 mg/ml LDH and 0.1 mg/ml pyruvate kinase in 50 mM potassium phosphate buffer, pH 7.0.

The specific activity of pyruvate kinase was 32.5 units/mg in water (0.1M potassium phosphate, 60 mM KCl:$MgSO_4.7H_2O$ (1:1), pH 7.5) where one unit of the activity consumes one μ mole of NADH per minute at 30°C.

IV.2.2.4 Spectroscopic Measurements

U.V.-visible absorption spectra of pyruvate kinase catalyzed

reaction system was recorded by Gilford Response U.V.-Vis. Spectrophotometer at 30 ± 0.2°C maintained by an external thermostat. The reference used in the case of reverse micellar solution was adjusted at the same water content as that of the sample solution. The extinction coefficient given for NADH in water and micellar solution was taken to be 6.2 x 10^3 $M^{-1}cm^{-1}$.

IV.3 Results and Discussion

After solubilization of the enzymes lactate dehydrogenase and pyruvate kinase into reverse micellar media of 4% cetrimide in $CHCl_3$-isooctane (1:1, v/v), the activities of these enzymes were optimized by the manipulation of various parameters like waterpool size, pH, surfactant concentration etc. in the reverse micellar solution.

In the reverse micelle's polar core, waterpool is the important region where the entrapped enzymes are protected from the unfavorable action of the organic solvents. The size of waterpool may be varied by changing either the water content or the surfactant concentration in the reverse micellar solution. First the activity of lactate dehydrogenase in cetrimide/$CHCl_3$-isooctane (1:1, v/v) micellar system was measured by using sodium pyruvate and NADH solution separately and then was optimized by changing all possible parameters.

IV.3.1 Activity of LDH in Reverse Micelles

Fig. IV.1 shows the effect of variation of water content

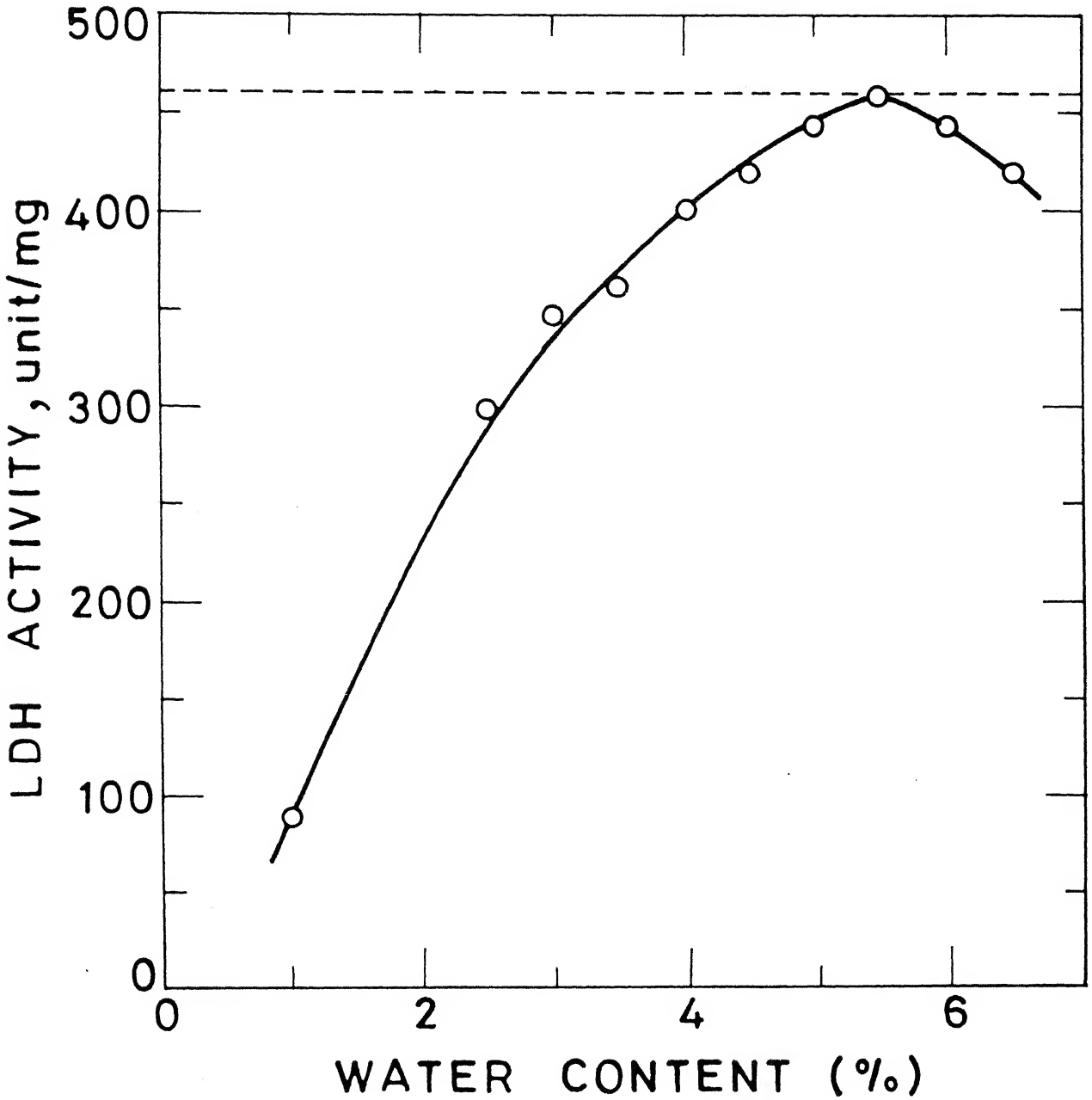


Fig. IV.1. Specific activity of lactate dehydrogenase as a function of water content in the reverse micelles of 4% cetrimide in chloroform-isooctane (1:1, v/v). The concentrations are as follows: [sodium pyruvate] = 1 mM, [NADH] = 0.2 mM and 0.1 M potassium phosphate buffer, pH 7.0.

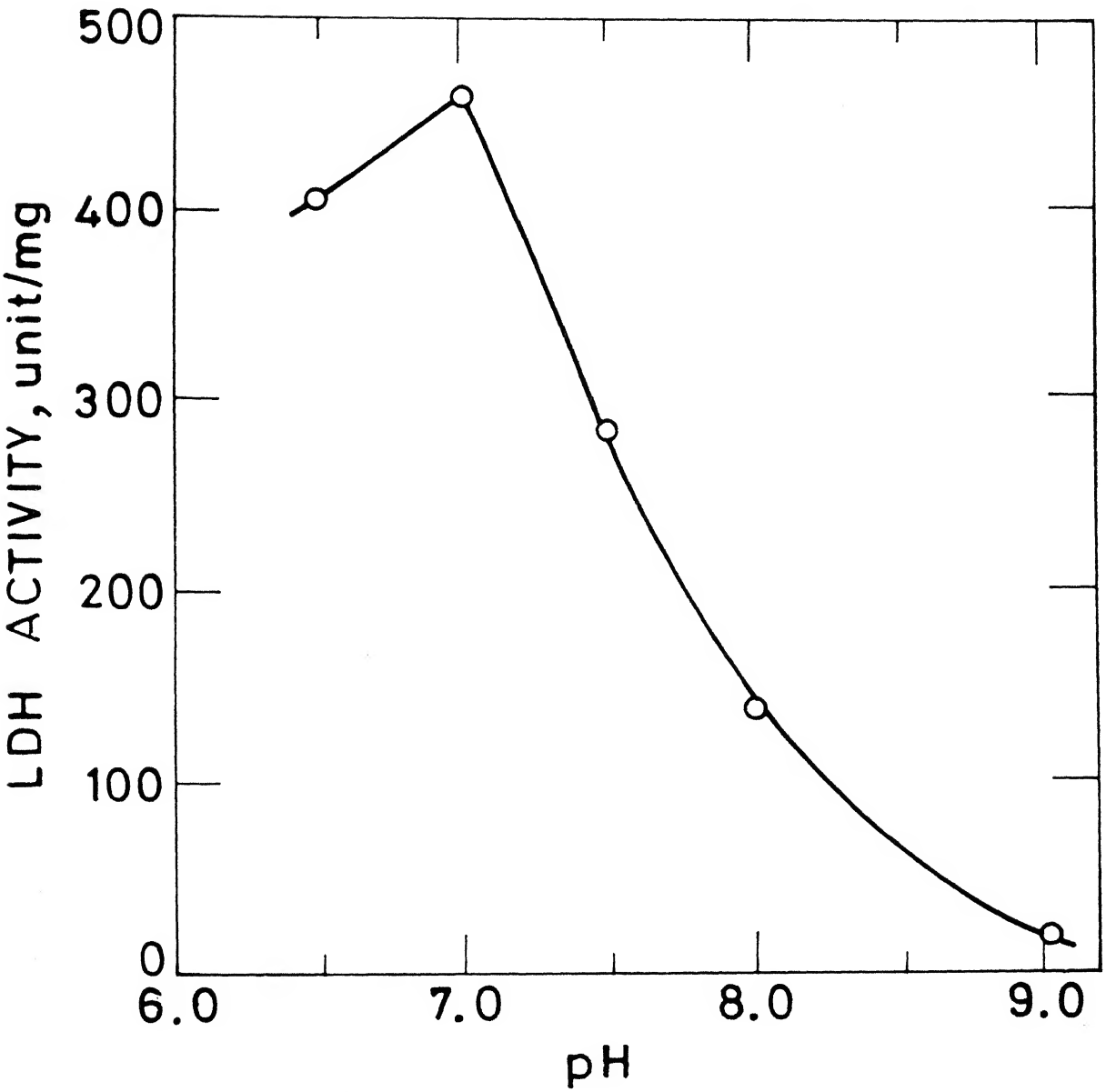


Fig. IV.2. Effect of pH on lactate dehydrogenase activity in 4% cetrimide/chloroform-isooctane (1:1, v/v) at 5.5% water content. Concentrations are: [sodium pyruvate] = 1 mM; [NADH] = 0.2 mM; [LDH] = 0.1 μg/ml.

(expressed in term of percentage of overall micellar solution) on the LDH activity in the reverse micellar solution of 4% cetrimide in $CHCl_3$-isooctane (1:1, v/v) at pH 7.0. At low initial water content (~ 0.6%), the enzyme activity was almost zero. An increase in water content enhanced the activity of LDH, reaching a maximum at 5.5% water content. The highest activity was observed in reverse micelles was equal to the activity obtained in aqueous buffer at pH 7.0 [21] as shown in Fig. IV.1. A waterpool size with water content in the range of 2 to 5.5% apparently achieved the most functional conformation of LDH in this new media with increased activity.

The pH dependence of lactate dehydrogenase activity in reverse micellar solution at 5.5% water content is shown in Fig. IV.2. The activity of LDH was highly regulated with variation in pH of the buffer solution of enzyme assay mixture transferred into the reverse micellar solution. The nature of dependence is similar to that found in the case of aqueous system. The maximum activity of LDH was observed at pH 7.0 in both the aqueous and reverse micellar solution. No pH_{opt} shift has been observed by transferring the reaction from aqueous medium to reverse micellar one in non-polar solvent mixture. A detailed investigation on LDH activity in CTAB/H_2O/$CHCl_3$-isooctane system has been reported elsewhere by Katiyar et al. [18].

Once the single component LDH was successfully solubilized in the cetrimide/H_2O/$CHCl_3$-isooctane reverse micelles, a

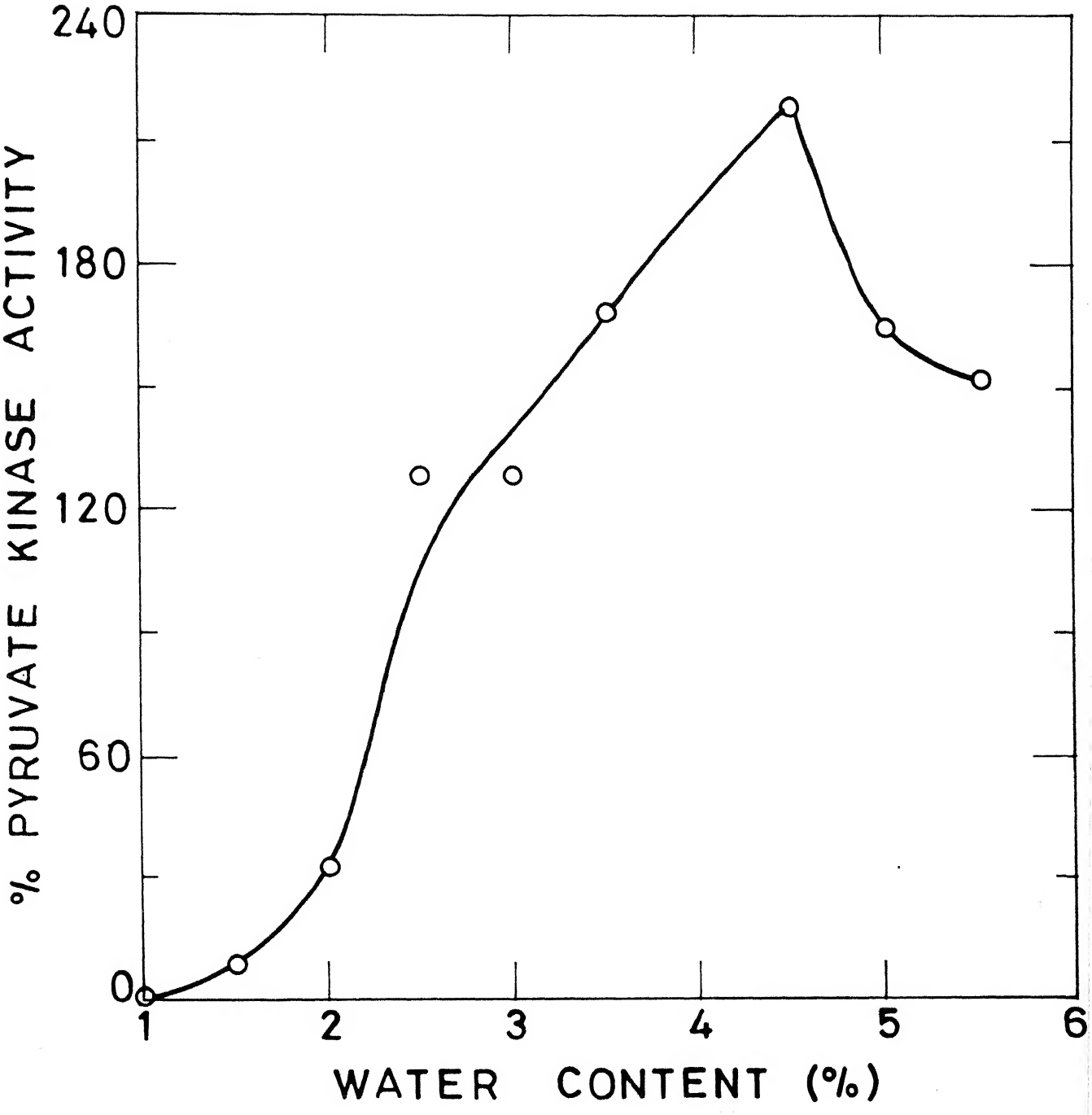


Fig. IV.3. Variation of Pyruvate Kinase activity with increasing water content in reverse micellar solution of 4% cetrimide in $CHCl_3$-isooctane (1:1, v/v) at pH 7.8. Concentrations are [phospho(enol)pyruvate] = 0.24 mM, [ADP] = 0.40 mM, [NADH] = 0.1 mM, [pyruvate kinase] = 0.2 μg/ml and [LDH] = 0.1 μg/ml.

systematic study was conducted on the determination of pyruvate kinase activity coupled with LDH in this system. As in the case of glutathione reductase and lactate dehydrogenase, effect of different parameters of reverse micellar media on activity was studied.

IV.3.2 Activity of Pyruvate Kinase in Reverse Micelles

IV.3.2.1 Effect of Water Content

Fig. IV.3 represents the variation of the activity of pyruvate kinase (expressed in term of percentage of control activity in aqueous solution at optimum condition) as a function of amount of water transferred inside the reverse micelles of cetrimide in $CHCl_3$-isooctane. A bell shaped curve was obtained which is similar to enzyme behavior reported by Luisi et al. [9,27-28] for α-chymotrypsin, lysozyme, horse liver alcohol dehydrogenase; Martinek et al. [11,13,29] for peroxidase,laccase, β-glutamyltransferase; Lee et al. [24] for cholesterol oxidase and Katiyar et al. [17-20,30] for glutathione reductase, dihydrofolate reductase, malate dehydrogenase, glucose-6-phosphate dehydrogenase etc. The activity of the enzyme increases with increase in water content from a low value 1% to 4.5% and again decreases with further increase in water content. At this water content pyruvate kinase in reverse micelles exhibits around 200% activity than that in aqueous solution. The phenomenon of superactivity may be attributed to the special microenvironment generated by forming right kind of surfactant aggregates where

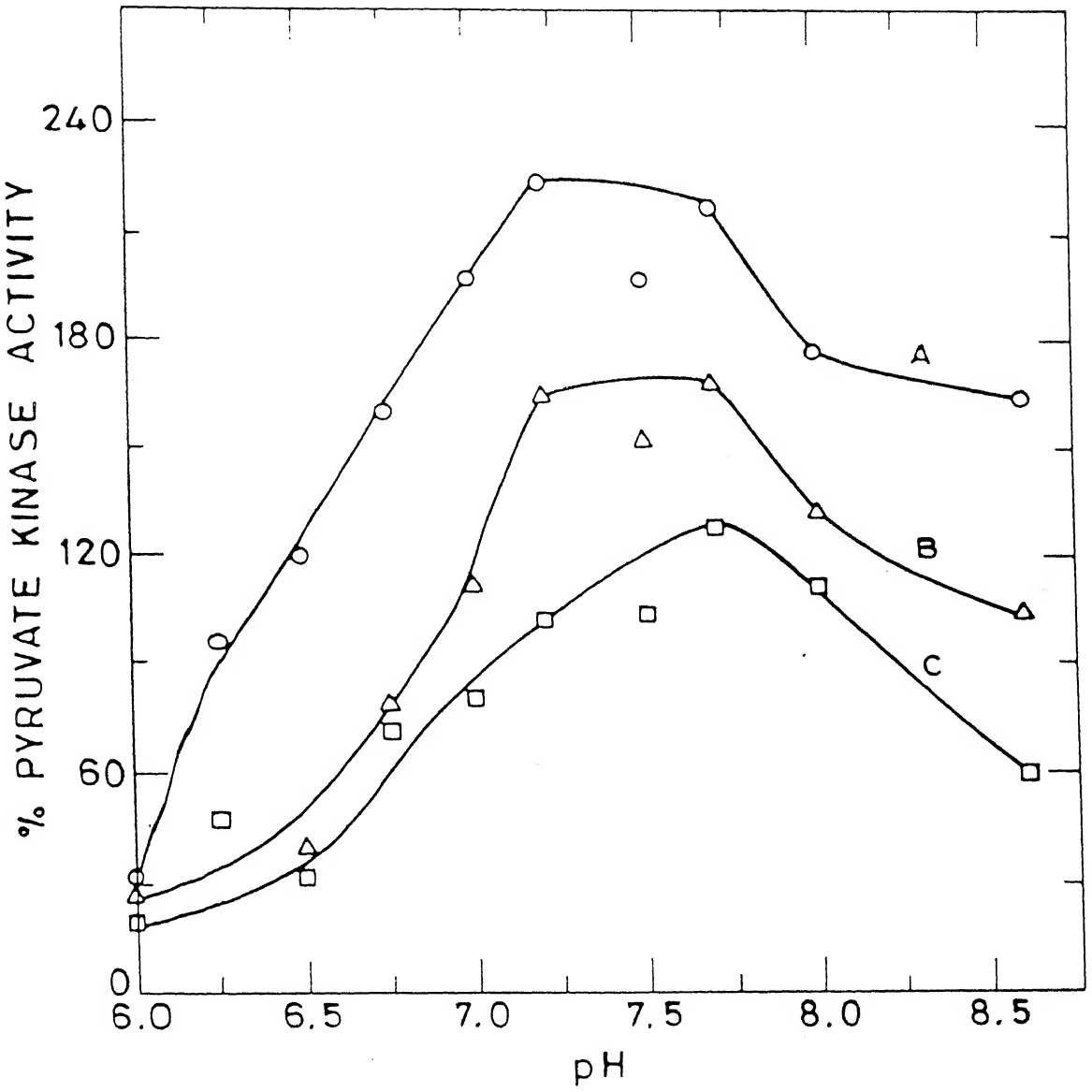


Fig. IV.4. Variation of pyruvate kinase activity as a function of pH at different water content in reverse micelles of 4% cetrimide in chloroform-isooctane (1:1, v/v) (□) 2.5%, (△) 3.5% and (○) 4.5%. Concentrations are: [PEP] = 0.24 mM, [ADP] = 0.4 mM [NADH] = 0.1 mM, pyruvate kinase = 0.2 μg/ml and [LDH] = 0.1 μg/ml.

the enzyme retains its most active conformation. Another reason for superactivity is such that the size of waterpool is favorable enough to suitably accommodate the enzyme molecules in their most active conformation at particular pH. It is of interest to note that the optimum value of waterpool size differs for different enzymes [31].

IV.3.2.2 Effect of pH

Investigation on the effect of pH on enzyme activity often provides interesting information. In order to determine the pH profile in reverse micellar solution, the buffer of the stock solution 50 mM potassium phosphate, 250 mM KCl:$MgSO_4.7H_2O$ (1:1) was used from pH range 6.0 to 8.6. Fig. IV.4 depicts the dependence of percent control of pyruvate kinase activity as a function of pH of the stock aqueous buffer transferred into the micellar solution of cetrimide in $CHCl_3$-isooctane (1:1, v/v) at three different values of water content 2.5, 3.5 and 4.5%. Almost similar trend of pH activity profile was obtained for each water content value where the enzyme activity first increases with increasing pH, achieves a maximum at optimum pH and then decreases. Pyruvate kinase shows maximum activity in the pH range from 7.2 and 7.8 in all three cases. The activity of pyruvate kinase was found to be greater (i.e. superactivity) in cetrimide/$CHCl_3$-isooctane at pH greater than 7.0 at high value of water content i.e. 3.5 and 4.5% of overall volume of assay mixture. Another point to be mentioned here is the loss of

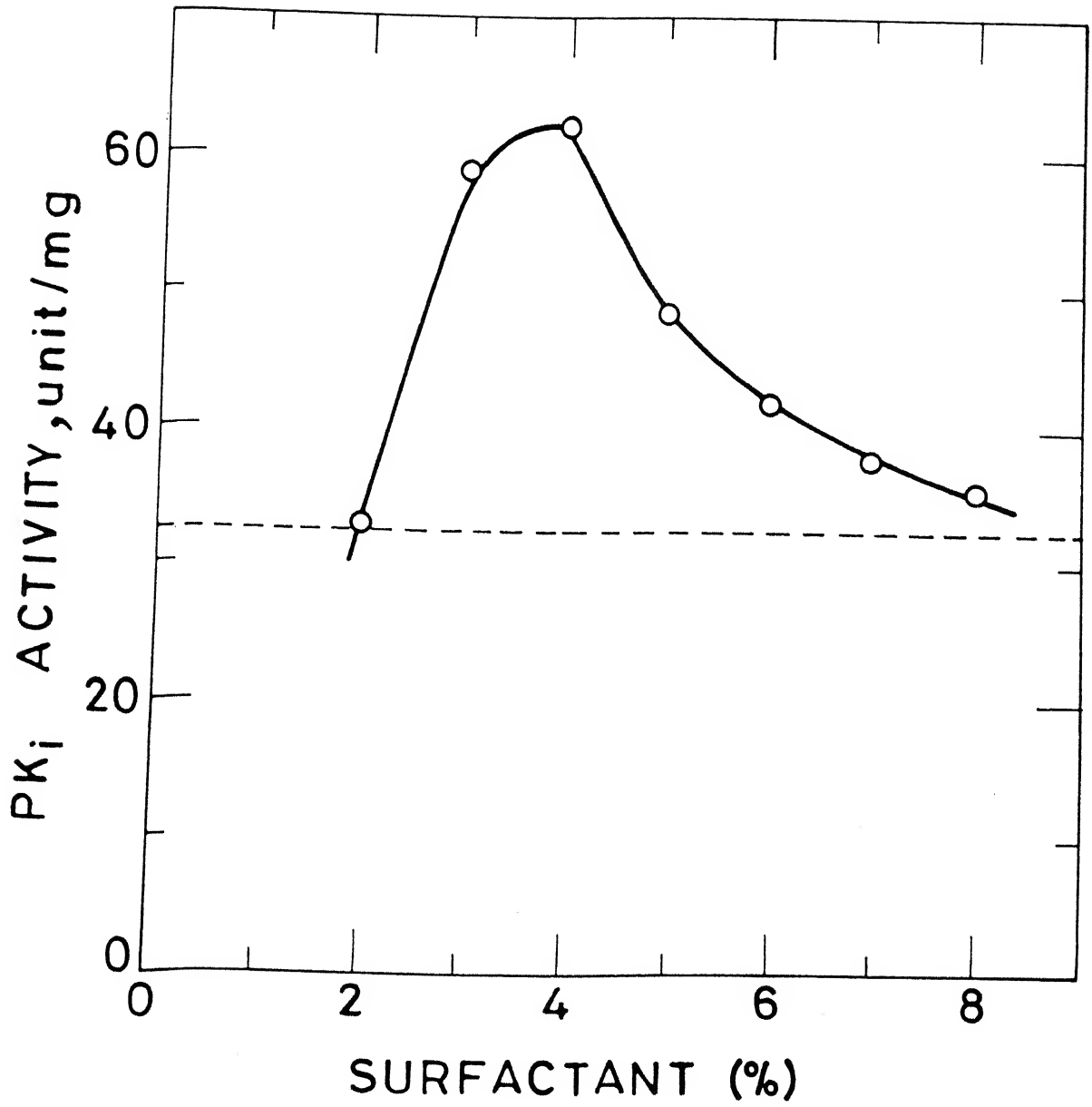


Fig. IV.5. Dependence of pyruvate kinase activity on the concentration of cetrimide in $CHCl_3$-isooctane (1:1, v/v) reverse micelles at 4.0% water content and pH 7.8. Concentrations are same as in the Fig. IV.4.

enzyme activity at pH values less than 7.0 which is also observed in the case of pH profile in aqueous solution. No significant shift in optimum pH of the activity of enzymes has been observed in transition from aqueous solution to reverse micellar solution. However, in general, alkaline shift in optimum pH of the enzyme is observed when the transfer of enzyme occurs from aqueous to reverse micellar medium [9,17]. One of the most important finding from this Fig.IV.4 is that at certain waterpool size, the enzyme (pyruvate kinase) exhibits superactivity (its activity is about two times more than in aqueous solution) in this cationic reverse micellar solution.

IV.3.2.3 Effect of Surfactant Concentration

The surfactant concentration plays very important role on the regulation of activity of the enzyme in micellar solution. For the display of maximum enzyme activity in reverse micelles, the optimum concentration of the surfactant is very critical. Effect of surfactant concentration on the rate of reaction catalyzed by pyruvate kinase was performed by keeping the ratio of the concentration of water and surfactant constant. Variation of concentration of cetrimide for pyruvate kinase activity showed that enzyme activity in reverse micelles was markedly regulated by this parameter. Fig. IV.5 presents the effect of change in concentration of cetrimide (expressed in term of percent) in the reverse micelles of cetrimide/H_2O/$CHCl_3$-isooctane (1:1, v/v) at fixed water content 4% and pH 7.8. The rate of enzyme reaction increases with the increase in the cetrimide concentration and

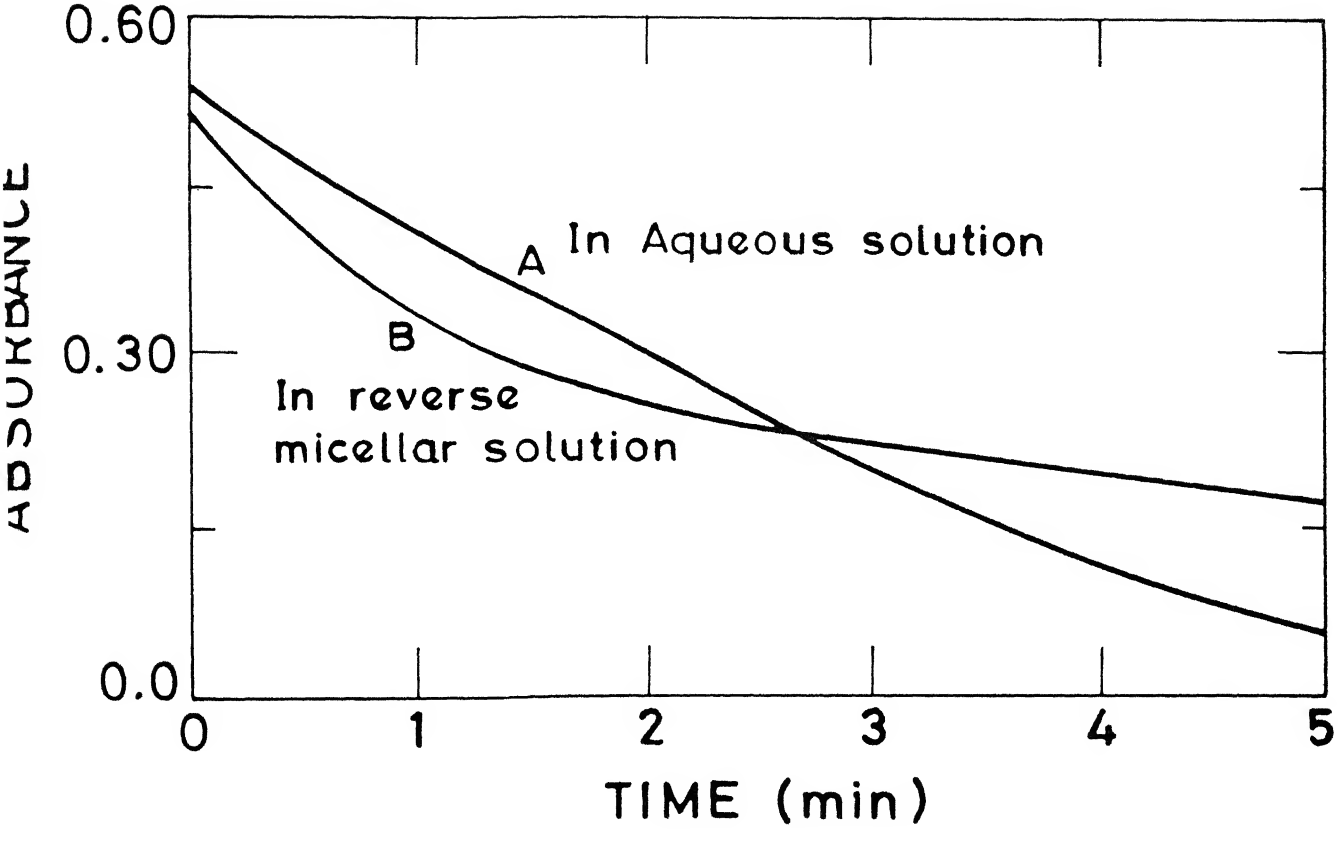


Fig. IV.6. Kinetic curve for consumption of NADH in the coupled enzyme system in [A] water and in [B] reverse micellar solution of 4% cetrimide in $CHCl_3$-isooctane (1:1, v/v) at 4% water content of 50 mM potassium phosphate, 60 mM KCl:$MgSO_4$ (1:1), pH 7.5. Experimental conditions are: [PEP] = 0.24 mM, [ADP]= 0.4 mM, [NADH] = 0.1 mM, [pyruvate kinase] = 0.2 μg/ml and [LDH] = 0.1 μg/ml.

attains maximum at 4% and then decreases with further increase in concentration, up to 8%. The substrate and enzyme reverse micellar solution was not optically transparent at room temperature at the surfactant concentration above 8% of overall micellar solution. This solution became very clear at temperature less than 25°C. At all the concentrations of substrate investigated, pyruvate kinase showed higher activity than that in water. The maximum activity achieved in the range of 3 to 4% was twice than the value in aqueous buffer at optimum condition. The enhanced activity of pyruvate kinase might be due to the incorporation of enzyme in its most active conformation under right kind of restricted microenvironment by aggregation of cationic surfactant in non-polar solvent of chloroform-isooctane (1:1, v/v).

IV.3.2.4 Absorbance with the Function of Time

A more clear observation regarding the behavior of this double enzyme system (pyruvate kinase/lactate dehydrogenase in both the reverse micellar and aqueous system is shown in Fig. IV.6. This figure shows the 'absorbance vs. time' kinetic curve for the consumption of NADH in the enzymic reaction. For comparison the data on the reaction in the same buffer without the surfactant and organic solvents are given. As in water [22] in reverse micellar system, there is a lag-period in the action of the double enzyme system. The initial velocity of pyruvate kinase lactate dehydrogenase catalyzed reaction in

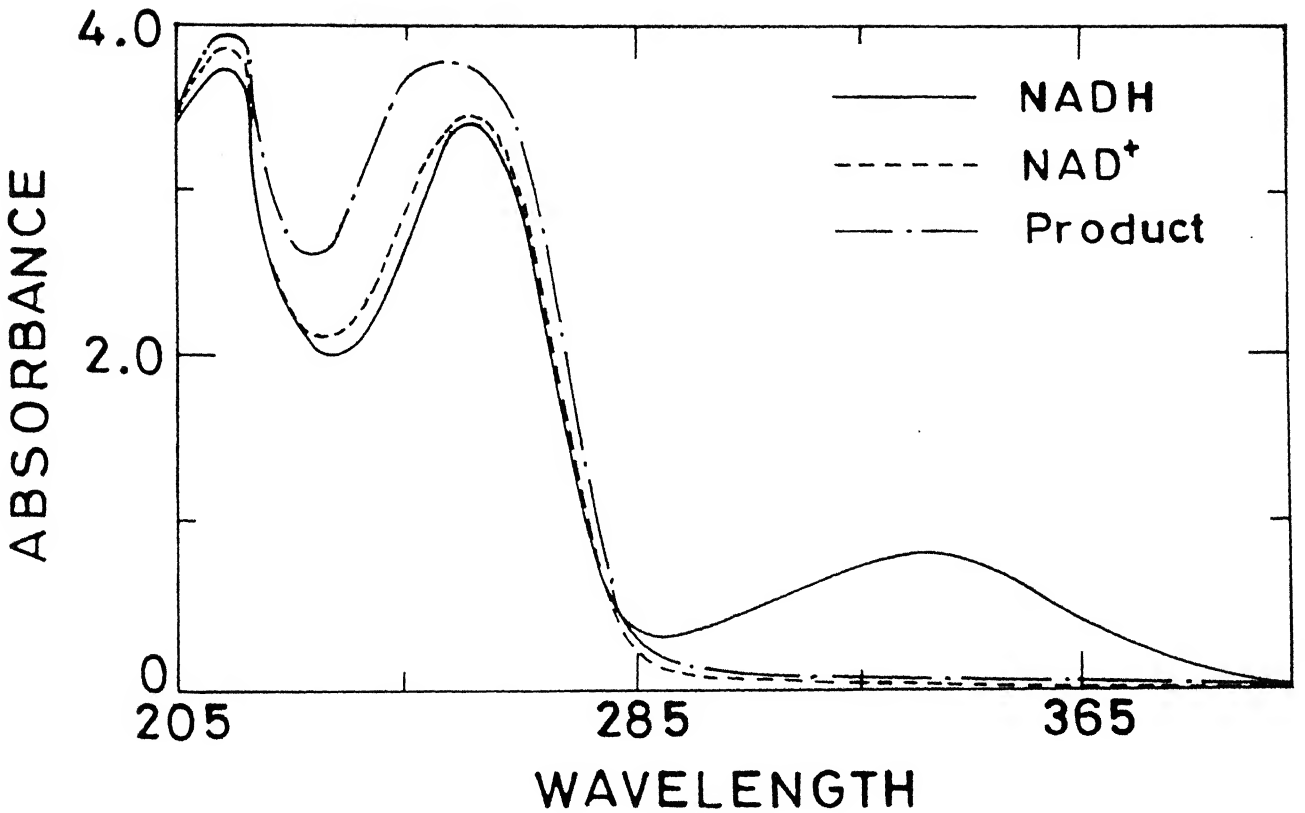


Fig. IV.7. Electronic absorption spectra of: (---) authentic NAD^+; (——) NADH containing reaction system before start of the reaction; and (·—·—) after the completion of coupled enzymes (pyruvate kinase + lactate dehydrogenase) catalyzed reaction in aqueous buffer (50 mM potassium phosphate, 60 mM KCl: $MgSO_4$, pH 7.8).

reverse micelles is faster than that in water, but the rate of reaction becomes almost zero, 3 minute after the start of reaction. This indicates poor stability of the enzyme in the reverse micellar system. However, this is not the case in the aqueous solution.

IV.3.3 Spectral Study of Combined Enzyme Catalyzed Reaction

The absorption spectra of enzymes containing reaction system is notably affected by changing the environment of reaction from aqueous to organic solution. Sometimes, significant perturbation in the spectral nature occurs which indicates the behavioral change of enzymes in new media. The U.V.-Visible spectral study of the enzyme reaction system was carried out in order to establish the fact whether the reactions in reverse micellar solution and in water are identical. For this purpose, the absorption spectra have been recorded before the start and after the completion of reaction respectively in both the media. Fig.IV.7 shows the absorption spectrum of authentic NAD^+ and NADH containing reaction system before start of the reaction. The nature of spectra of the reaction system (after the completion of enzymatic reaction) is similar to that of the authentic NAD^+. These observations show the formation of NAD^+ from NADH after the addition of enzymes in reaction components in the presence of other species in aqueous solution. Similar absorption spectra have been obtained in the case of cetrimide/H_2O/$CHCl_3$/isooctane (1:1, v/v) reverse micellar system with a slight change in the

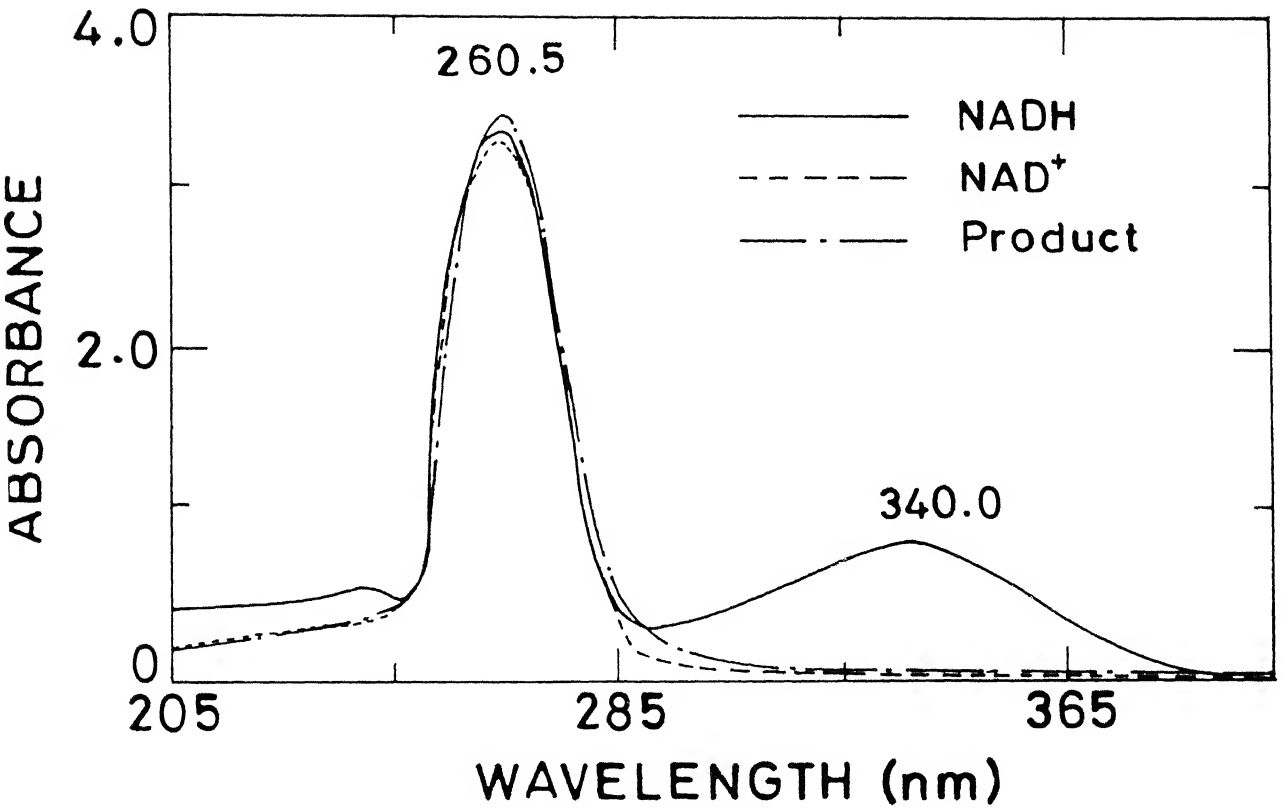


Fig. IV.8. Electronic absorption spectra of (– – –) authentic NAD^+, (——) NADH containing reaction system before start of the reaction and (·—·-) after the completion of coupled enzymes (pyruvate kinase + lactate dehydrogenase) catalyzed reaction in cetrimide/ $CHCl_3$-isooctane (1:1, v/v) at 4% water content of 50 mM potassium phosphate buffer, pH 7.8.

absorption peaks as shown in Fig. IV.8. In the aqueous solution the absorption maxima of NADH containing reaction system occurs at 253 nm and 340 nm whereas NAD^+ (authentic sample) and the product after the completion of enzyme reaction show their absorption peaks at 266 nm. The characteristic peak of NAD^+ in authentic sample and in the product after the completion of enzyme reaction in cetrimide/$CHCl_3$-isooctane (1:1, v/v) solution is slightly shifted to 260.6 nm. The blue shift by 5 nm in the absorption peak of NAD^+ in reverse micellar media may occur due to little perturbation in the nicotinamide containing ring in this new media. However, these observations indicate that the decrease in absorbance at 340 nm during the assay is solely due to the enzyme oxidation of NADH to NAD^+ with the simultaneous conversion of phospho(enol)pyruvate to pyruvate in presence of ADP by the catalysis of enzymes. The similar nature of spectrum in aqueous and reverse micelles established the similarity of enzyme reaction in both the media.

IV.3.4 Kinetic Characteristics

IV.3.4.1 Effect of Enzyme Concentration

Under the usual <u>in vitro</u> assay conditions, the enzyme is present in limiting or 'catalytic' amounts. The $[E]_{ov}$ is generally very very less (i.e. 10^{-12} to 10^{-7}M) in comparison to $[S]_{ov}$ (i.e. 10^{-6} to 10^{-2}M). At any substrate concentration the instantaneous or initial velocity is given by:

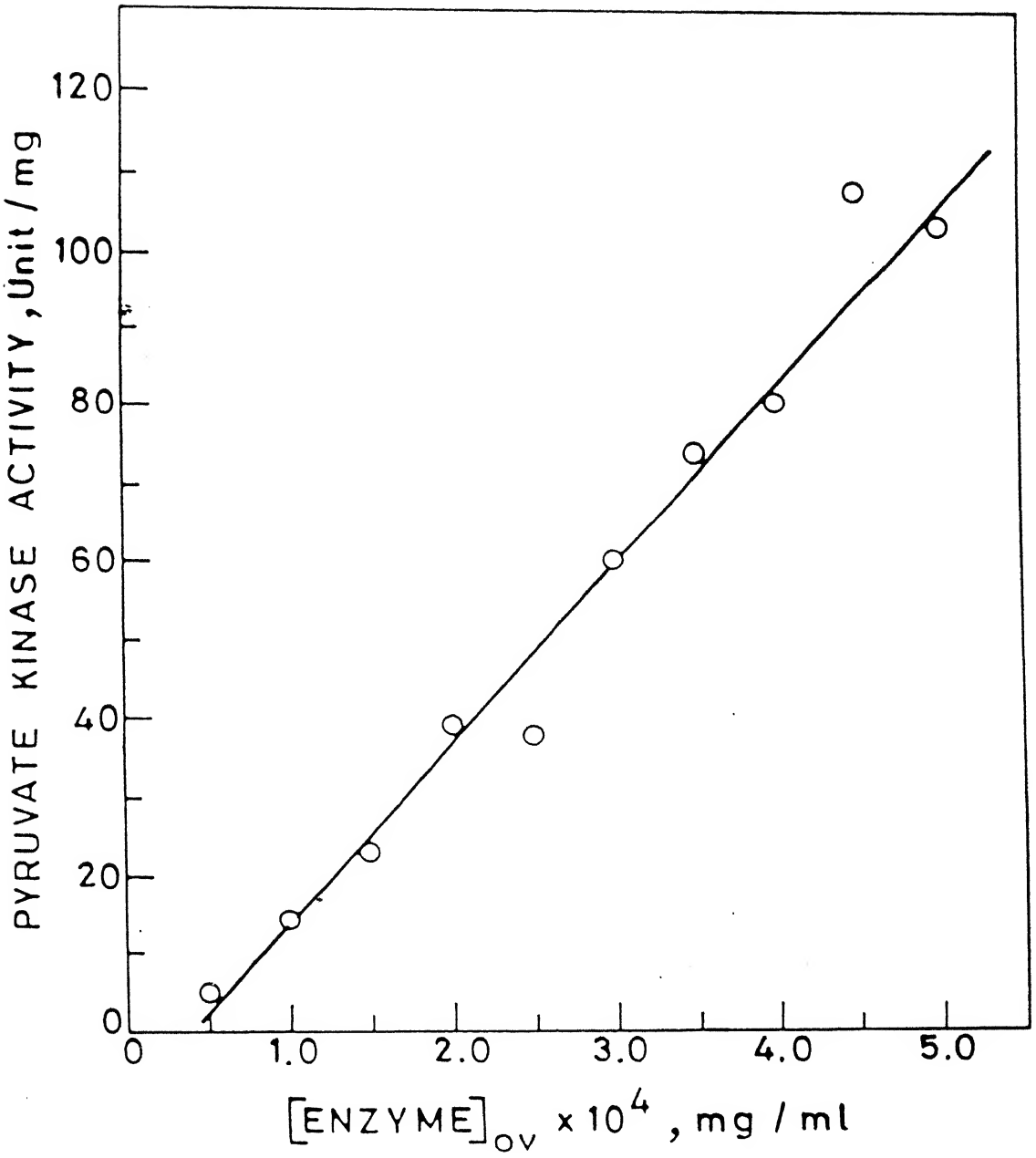


Fig. IV.9. Dependence of initial rate of pyruvate kinase catalyzed reaction on the concentration of enzyme in cetrimide/$CHCl_3$-isooctane (1:1, v/v) reverse micellar solution at 3% water content, pH 7.8. Concentrations are: [PEP] = 0.24 mM, [ADP] = 0.4 mM, [NADH] = 0.1 mM and [LDH] = 0.1 μg/ml. Buffer used was 50 mM potassium phosphate, 60 mM KCl:$MgSO_4$ (1:1).

$$v = \frac{[S]V_{max}}{K_M+[S]} = \frac{[S]K_p[E]_t}{K_M+[S]} = \frac{K_p}{(1 + K_M/[S])}[E]_t \quad \ldots \text{ IV.3}$$

Thus the initial velocity is directly proportional to enzyme concentration at all substrate concentrations, and this fact is used to quantitate the concentration of enzyme in any preparation, at any stage of purification. The striking point of this observation is that the relationship between the initial rate of enzyme reaction and concentration of enzyme is linear only if true initial velocities are measured, that is the rate of product formation or transfer of substrate to product must be constant over the entire time interval of the assay. Fig. IV.9 shows the change of initial velocity of the coupled enzymes catalyzed reaction at different concentrations of pyruvate kinase and at fixed concentrations of phospho(enol)pyruvate (0.24 mM), ADP (0.4 mM) and NADH (0.1 mM) in the reverse micellar solution of 4% cetrimide in $CHCl_3$-isooctane (1:1, v/v) at 3% water content and pH 7.8. The linear nature of the plot indicates thereby that the molecules of enzyme act independently in reverse micellar solution. This behavior of pyruvate kinase coupled with lactate dehydrogenase is indicative of the homogeneity of reverse micellar solution and purity of enzymes.

IV.3.4.2 Effect of Substrate Concentration

At a constant concentration of enzyme, the reaction rate

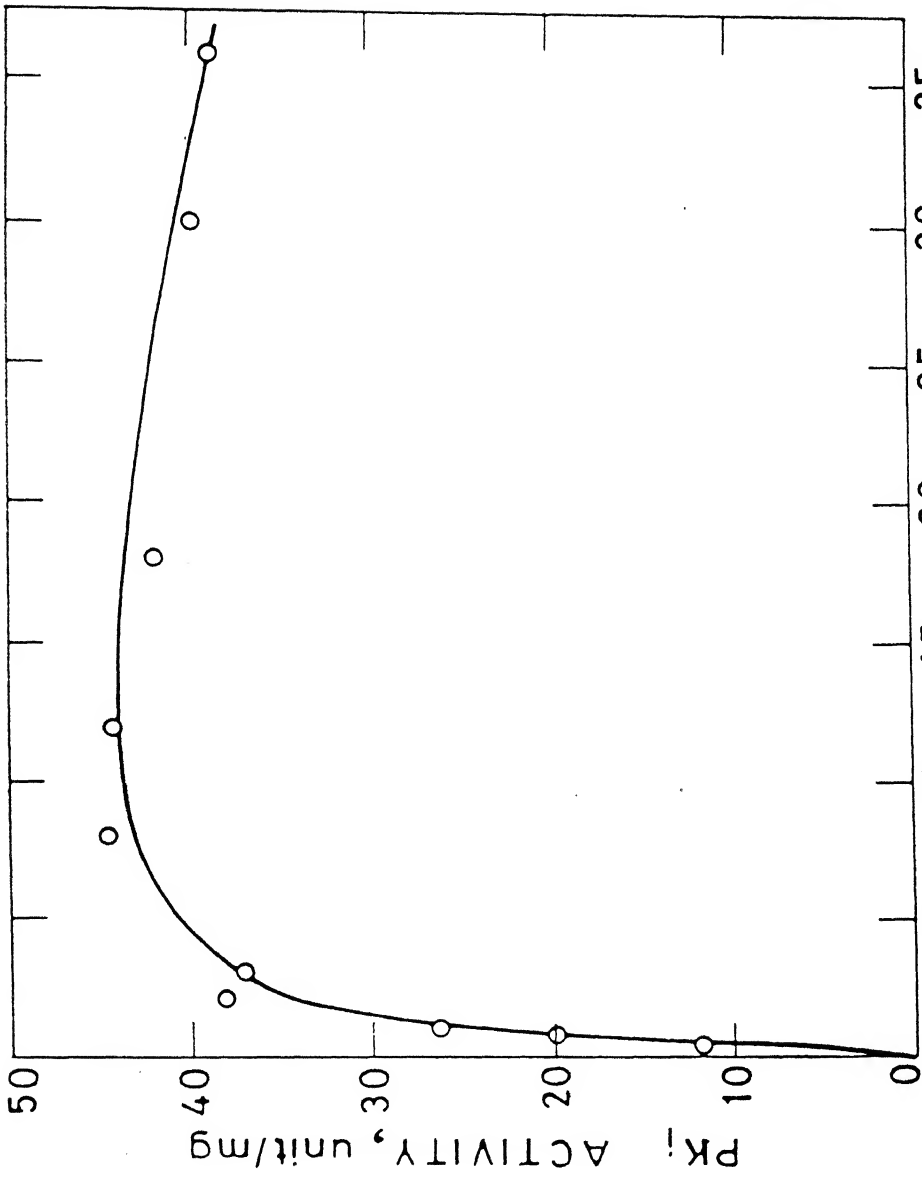


Fig. IV.10. Effect of phospho(enol)pyruvate concentrations on pyruvate kinase activity in the reverse micellar solution of 4% cetrimide in $CHCl_3$-isooctane (1:1, v/v) at 4% water content, pH 7.8. Concentration are: [ADP] = 0.4 mM, [NADH] = 0.1 mM, [pyruvate kinase] = 0.2 μg/ml and [LDH] = 0.1 μg/ml.

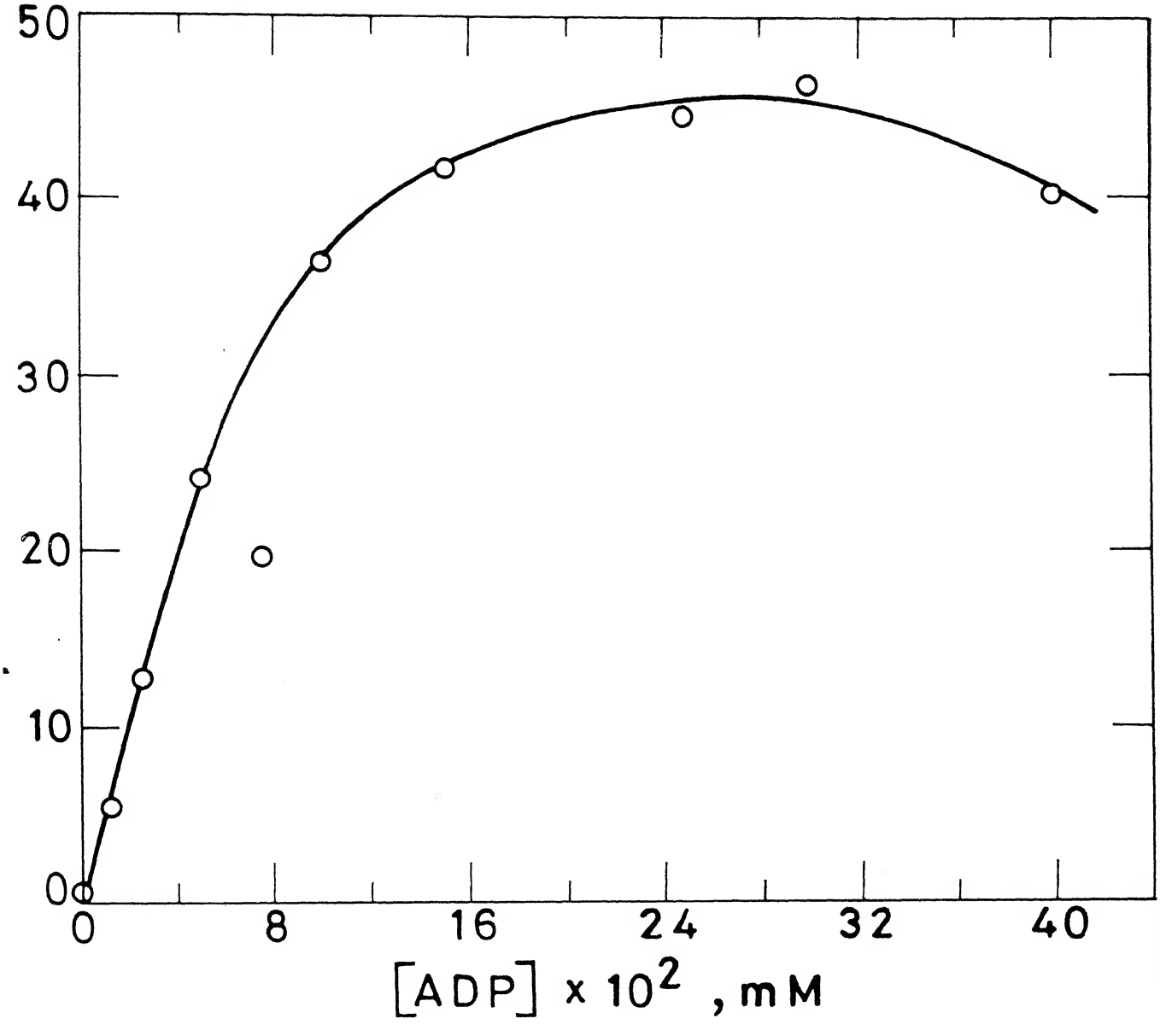


Fig. IV.11. Effect of ADP concentration on pyruvate kinase activity in cetrimide/$CHCl_3$-isooctane (1:1, v/v) reverse micellar solution at 4% water content, pH 7.8. Concentrations are: [PEP] = 0.24 mM, [NADH] = 0.1 mM, [pyruvate kinase] = 0.2 μg/ml and [LDH] = 0.1 μg/ml.

increases with increasing substrate concentration until a maximal velocity is reached. In contrast, uncatalyzed reactions do not show this saturation effect. As in the case of aqueous solution, the condition for the maximal activity of pyruvate kinase catalyzed reaction in cetrimide/$CHCl_3$-isooctane (1:1, v/v) reverse micellar solution was optimized by changing the concentration of PEP and ADP on the activity of coupled enzyme system. Fig. IV.10 shows the effect of PEP concentration on pyruvate kinase activity at fixed concentration (0.4 mM) of ADP, (0.1 mM) of NADH in cetrimide/$CHCl_3$-isooctane (1:1, v/v) solution at 4% water content, pH 7.8. Up to the concentration of 50 mM, the rate of reaction is almost linearly proportional to [PEP]. Above 100 μM of [PEP], the enzyme pyruvate kinase follows saturation kinetics at the experimental condition specified in legends of Fig. IV.10. At very high [PEP], pyruvate kinase coupled reaction system shows slight inhibition in the rate of reaction. Similar nature of hyperbolic plot has been observed with variable concentration of ADP at fixed concentrations of other substrates. Fig. IV.11 shows the dependence of pyruvate kinase activity on the concentration of ADP at 0.4 mM PEP and 0.1 mM NADH at 4% water content of pH 7.8 in reverse micellar solution. In both the cases i.e. in Figs. IV. 10 & 11, pyruvate kinase in micellar solution follows the Michaelis-Menten Kinetics within the specified conditions. At lower concentration, linearity is observed whereas slight inhibition in enzyme activity is observed at very high substrate concentration. The hyperbolic nature of curve is identical to that found in the case

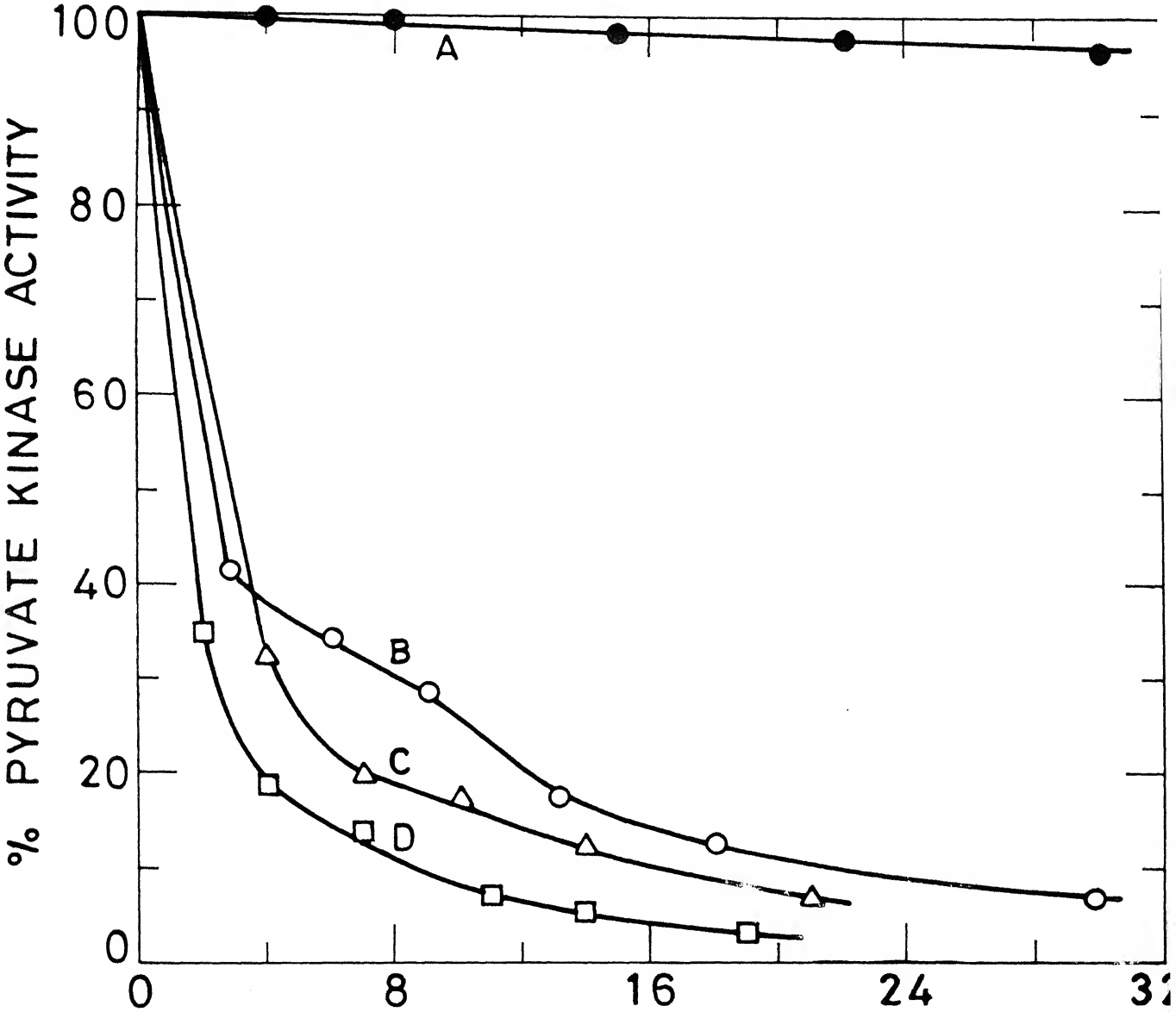


Fig. IV.12. Stability of pyruvate kinase as a function of incubation time of enzyme in aqueous and 4% cetrimide in chloroform-isooctane (1:1, v/v) reverse micellar solution at different conditions: water [A] and water content; [B] 3.5%, [C] 4.0% & [D] 5.0%. Buffer used 50 mM potassium phosphate, pH 7.5.

of aqueous solution.

IV.3.5 Time Dependent Stability

Often the stability of the enzyme is determined in order to find out the storage conditions in the convenient way. Continuous use is especially important in maintaining a constant environment for the used biocatalyst and for the economical process, a factor which is very important in maintaining enzyme stability. Thus stability of enzymes in reverse micelles containing organic solvents is reflected in the wider use at their potentialities in this hostile media in large scale process. Good storage stability is of great importance, owing to the obvious difficulties for use in biotechnology due to the denaturation or continuously decreasing activity due to contact with non-polar solvents. Fig. IV.12 shows the time dependent stability in term of the percentage residual activity as a function of incubation time of enzyme (pyruvate kinase) in water and reverse micellar solution of cetrimide/H_2O/$CHCl_3$-isooctane (1:1, v/v). Control in stability of the enzyme was determined at zero elapsed time of incubation of enzyme in both the aqueous and micellar system under similar experimental conditions. The time dependent stability of pyruvate kinase was significantly affected by the change in the percent of water content in reverse micelles. Plot A is the case of water, where the enzyme shows almost constant activity over the wide range of period. However, the residual activity of pyruvate kinase is decreased sharply in

reverse micellar solution with the incubation time at specified experimental condition i.e. 3.5 to 5.0% water content at pH 7.5. The decrease in stability of the enzyme is slow with increased water content of reverse micellar waterpool. Around 80% activity of the enzyme was lost even after half an hour of incubation in reverse micelles. Thus pyruvate kinase shows poor stability in the specified experimental condition of cetrimide/$CHCl_3$-isooctane (1:1, v/v) reverse micellar system. Nevertheless, the storage stability can be enhanced by changing the different parameters like water content, pH, temperature, concentration of surfactant and nature of solvents etc. in reverse micellar system. The present study shows that small amount of water present in reverse micellar core can regulate the storage stability of enzyme. Addition of substrate PEP, ADP and coenzyme NADH could not improve the residual activity of enzyme in both aqueous and reverse micellar media. Thus, unlike the case of glutathione reductase discussed in chapter III, the same experimental condition at which pyruvate kinase shows super activity in reverse micellar solution, is not suitable for the storage stability in this media.

The activity of the coupled enzyme system in reverse micelles in non-polar solvents depends upon the activity of pyruvate kinase and lactate dehydrogenase separately and together with in the same media. Besides this the added reagents must of course be capable of acting in the same condition (pH, temperature etc.) as the enzyme being measured and must not

interfere with the enzyme's activity. All these factors make the study of multi-enzyme system difficult in reverse micellar media. There are very sparse examples for the exhibition of superactivity of very big and complex enzymes (i.e. very high mol. weight with large sub units) in reverse micelles. Moreover, this phenomenon is almost rare in the case of coupled or multi-enzyme systems, studied in this novel unique milieu. In this regard, the reverse micellar waterpool of cationic surfactant cetrimide containing the solvent system of $CHCl_3$-isooctane (1:1, v/v) has proven to be a novel media for the study of this double enzyme system <u>in vitro</u>. Both the enzymes namely pyruvate kinase and lactate dehydrogenase follow the Michaelis-Menten kinetics almost similar to that observed in aqueous solution. However, this reverse micellar system has not proven to be a suitable viable medium for the storage of these enzymes system, nevertheless the study is not limited and optimum condition can be investigated by changing different parameters for finding better storage condition.

IV.4 Conclusion

The study demonstrates how it is possible to build up a mini-metabolic sequence in order to assay an enzyme in conjugation with other enzyme in water-restricted microcaptive environment in non-polar solvents. In summary, the investigation of the catalytic role of the combined enzyme system in organic micellar media is a major step towards understanding of experimental techniques for carrying out multi-step enzyme catalyzed reactions

in organic solvents. These studies provide optimism for the use of reverse micelles as a versatile medium for a series of transformations catalyzed in a sequence by many enzymes in organic solvents. Under certain conditions, the complete metabolic cycle may be studied in this new microenvironment inside the bulk non-aqueous solvents through the use of suitable surfactants, co-surfactants and proper choice of organic solvents or by their suitable combinations. As there is no general theoretical formulation for the activity expression of enzymes in this media, the satisfactory progress is dependent on 'trial and error' process.

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CONCLUSION

(1) The extraction behavior of proteins in cationic and anionic reverse micellar systems via solid extraction method and liquid-liquid phase transfer method is markedly affected by the characteristics of proteins and external parameters (like pH, salt, surfactant concentration etc.) which govern the different type of interactions between proteins and micelles.

(2) The maximum extraction power of AOT/isooctane system for the studied proteins in general occurs at W_o value ~6 to 8 and for CTAB/$CHCl_3$-isooctane at W_o ~ 8 to 20 respectively. This unusual solubilization phenomenon of proteins in reverse micelles at low W_o indicates that solubilization capacity is imparted by novel physical properties of the water of the waterpool in comparison to bulk water.

(3) For the maximum solubilization of proteins, at least in the case of anionic surfactant AOT, the protein has to be positively charged. Similarly for the maximum solubilization of proteins in cationic surfactant CTAB protein should be negatively charged.

(4) Several binary, ternary and quarternary mixtures of different enzymes/proteins and other biomolecules have been successfully resolved into single components with the help of

a novel technique of selective and controlled solubilization of proteins in reverse micelles.

(5) Flavo enzyme glutathione reductase from two sources (namely Baker yeast and Bovine Intestinal Mucosa), a large enzyme (Mol. wt. 100,000 dalton and 2 subunits) has been successfully solubilized in CTAB cationic reverse micelles in the mixture of $CHCl_3$-isooctane (1:1, v/v). The activity of the enzyme was notably affected by different parameters like pH, W_o, surfactant and substrate concentrations etc.

(6) The enzyme exhibits almost the same activity in non-polar medium compared to that in aqueous buffer. The striking feature of yeast glutathione reductase solubilized in reverse micelles is that the enzyme retains its 80% activity even up to a month which is comparable to that in aqueous solution.

(7) The enzyme obeys the Michaelis-Menten Kinetics in both the aqueous and reverse micellar solution. Glutathione reductase from yeast follows the sequential mechanism in both the media. $K_{M,ap}$ values in reverse micellar and aqueous media are found to be close.

(8) The study on the coupled enzyme system (Pyruvate Kinase & Lactate dehydrogenase) in water restricted captive environment in non-polar solvents in presence of cationic surfactant is one of the major steps towards the understand-

ing of the experimental techniques to design and carry out multi-step biochemical transformations in membrane like environment.

(9) Despite the fact that these enzymes (Pyruvate Kinase, mol. wt. 2,40,000 dalton, 4 sub units; and lactate dehydrogenase, mol. wt. 1,45,000 dalton, 4 sub units) are relatively bigger in size and have more complex oligomericity, the coupled enzyme system shows the super activity i.e. the activity in reverse micellar solution is around two times greater than that in aqueous solution at optimum condition. However, cetrimide/$CHCl_3$-isooctane (1:1) is not a suitable medium for the storage of these enzymes as pyruvate kinase and lactate dehydrogenase both show very poor time dependent stability.

(10) Both the enzymes pyruvate kinase and lactate dehydrogenase follow the Michaelis-Menten kinetics in the non-polar reverse micellar media.

The first part of the work reports the study on the solubilization of enzymes/proteins and other biomolecules in reverse micellar media with the help of solid extraction and liquid-liquid phase transfer method. The selective and controlled forward and backward extraction of proteins from aqueous phase to reverse micellar phase and vice versa at different conditions have been exploited to generate a novel quantitative separation technique for the isolation of different proteins from mixtures. This liquid-liquid extraction process for the separation of

various proteins and biomolecules can be utilized for large scale, continuous and economical bioprocess technology.

In the second part of the work, we have successfully established the conditions under which glutathione reductase maintains almost the same activity as well as time dependent stability in reverse micellar medium as in aqueous medium. The combined enzyme system comprised of pyruvate kinase and lactate dehydrogenase showed higher activity in reverse micellar solution than that of aqueous solution. The present studies may help in devising suitable experimental process for the use of different class of enzymes/proteins in non-polar solvents for biotechnological applications.

LIST OF RESEARCH PUBLICATIONS:

1. Behaviour of enzymes in reverse micelles in non-aqueous solvents.
S.S. Katiyar, Anil Kumar, and Ajay Kumar (1988) Proc. Indn. Natl. Sci. Acad. 54, A, No.5, 711-716.

2. Reverse micelles as a versatile medium for the study of lactate dehydrogenase in vitro.
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3. Reverse micellar enzymology and its biotechnological applications.
S.S. Katiyar, Anil K. Awasthi and Ajay Kumar (1989) SUJST Vol.IX, 6-11.

4. Activity and kinetic characteristics of glutathione reductase in vitro in reversed micellar waterpool.
Ajay Kumar, Anil Kumar and S.S. Katiyar (1989) Biochim. Biophys. Acta 996, No. 1/2, 1-6.

5. The phenomenon of superactivity in dihydrofolate reductase entrapped inside reverse micelles in apolar solvents.
S.S. Katiyar, Anil Kumar and Ajay Kumar (1989) Biochemistry International vol. 19 No. 3, 547-552.

6. Stabilization and kinetic behavior of dihydrofolate reductase in microheterogeneous medium comprising of surfactants in non-aqueous system.
Anil Kumar, Ajay Kumar and sarvagya S. Katiyar (1989) Biochim. Biophys. Acta (Paper communicated)

7. Study of malate dehydrogenase in micocaptive environment generated by surfactants in apolar solvents.
Anil Kumar, Ajay Kumar and S.S. katiyar (1989) Eur. J. Biochem. (Paper communicated).

8. Catalytic efficiency of glutathione reductase in apolar solvents.
Ajay kumar, Anil Kumar and S.S. Katiyar (manuscript under preparation)

9. Catalytic efficiencies and kinetic properties of a coupled enzyme system (pyruvate kinase & lactate dehydrogenase) in the water restricted environment of non-aqueous solvents.
Ajay Kumar and S.S. Katiyar (manuscript under preparation).

10. Extraction and separation of enzymes and other proteins using reverse micelles.
Ajay Kumar and S.S. Katiyar (manuscript under preparation).

LIST OF PAPERS PRESENTED IN CONFERENCES

1. Catalytic efficiency of glutathione reductase in apolar solvents. Ajay Kumar and S.S. Katiyar
(24th Annual Convention of Chemists at Shivaji university, Kolhapur, India, 1987)

2. Behaviour of enzymes in reverse micelles in non-aqueous solvents. S.S. Katiyar, Anil K. Awasthi and Ajay Kumar
(Symposium on EMERGING FRONTIERS OF CHEMISTRY at BARC Bombay, India)

3. Reactions in non ionic reverse micelles.
Ajay Kumar and Sarvagya S. Katiyar
(IIIrd National conference on Surfactants, Emulsions and Biocolloids, Aligarh muslim university, India, 1987)

4. Catalytic activity of glutathione reductase and malate dehydrogenase in inverted micelles in non-aqueous solvents.
S.S. Katiyar, Anil K. Awasthi and Ajay Kumar
(IIIrd National conference on Surfactants, Emulsions and Biocolloids, Aligarh, India, 1987)

5. Reverse micellar system is a versatile medium for the enzyme catalysis and separation of complex protein mixture.
S.S. Katiyar and Ajay Kumar
(IVth National conference on Surfactants, Emulsions and Biocolloids, Bombay, India, 1989)

VITAE

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